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PREPARATION FROM HUMAN RED CELLS OF A SUBSTANCE INHIBITING VIRUS HEMAGGLUTINATION*

By PATRICK M. DEBURGH,‡ M.D., PEN-CHUNG YU§ M. B., CALDERON HOWE, M.D., AND MAX BOVARNICK, M.D.

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CORRECTION

Vol. 86, No. 5, November 1, 1947

Page 417, 9th line, the last sentence should read: *Two developed typical gastroenteritis, indicating that the agent had multiplied in cases 8 and 10, and was not a toxin ingested by the original cases.*

[illegible] under similar conditions in the elution phenomenon described by Hirst (2).

Recently three other laboratories have reported interesting data in relation to the effect of various tissue and cell extracts and other substances on virus hemagglutination and multiplication. Friedewald, Miller, and Whatley (3) described the hemagglutination-inhibiting effects of saline extracts of various human and animal tissues and red cells. Horsfall and McCarty (4) produced evidence to show that certain bacterial and plant polysaccharides seem capable of interrupting the multiplication of PVM virus in mouse lung. The intrapulmonary multiplication of virus was reflected by a rise in hemagglutination titer of triturated infected mouse lung. Administration of the polysaccharides diminished or prevented this increase in titer after infection. Green and Woolley (5) have demonstrated the inhibitory effect of various animal and

* Aided by a grant from the National Foundation for Infantile Paralysis.

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Experiments on the inhibiting effect of human and sheep red blood cell extracts on the hemagglutinating action of mumps and influenza viruses have been described in a previous report (1). Results were presented which suggested that the inhibiting agent found in these red cell extracts was a derivative of, or identical with, the material which in the intact cell constitutes the receptor site for the virus in the hemagglutination reaction. These results consisted partly in a demonstration that, in the systems studied, the species specificity of the inhibition reaction paralleled that of the hemagglutination reaction. Thus it is known that influenza (PR8) virus will agglutinate human and chicken red cells, but not sheep cells; and that mumps virus will agglutinate all three types of red cells. It was found that the human red cell extracts inhibited the agglutination of human and chicken cells by influenza virus; and that of human, chicken, and sheep red cells by mumps virus. Extracts of sheep cells, however, inhibited the agglutination of sheep cells by mumps virus, but did not inhibit agglutination of human and chicken cells by influenza virus. Further support for the view above mentioned was provided by the discovery that in a mixture of virus and inhibitor at 37°C. the latter was inactivated, just as is the receptor of intact cells under similar conditions in the elution phenomenon described by Hirst (2).

Recently three other laboratories have reported interesting data in relation to the effect of various tissue and cell extracts and other substances on virus hemagglutination and multiplication. Friedewald, Miller, and Whatley (3) described the hemagglutination-inhibiting effects of saline extracts of various human and animal tissues and red cells. Horsfall and McCarty (4) produced evidence to show that certain bacterial and plant polysaccharides seem capable of interrupting the multiplication of PVM virus in mouse lung. The intrapulmonary multiplication of virus was reflected by a rise in hemagglutination titer of triturated infected mouse lung. Administration of the polysaccharides diminished or prevented this increase in titer after infection. Green and Woolley (5) have demonstrated the inhibitory effect of various animal and

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plant polysaccharides on the hemagglutination of chicken red cells by influenza PR8 virus, and on the multiplication of the same virus in the chicken embryo.

The inhibitor previously reported from this laboratory was designated as a lipid extract of red cells. The present report deals with further experiments on methods of preparation of the soluble inhibitor, on its partial purification, and on some chemical properties of the most active preparations that we have thus far obtained.

EXPERIMENTAL

Assay.—For assay purposes one unit of inhibitor was designated as that amount of material which would completely inactivate two minimal hemagglutinating doses of influenza virus, strain PR8. The minimal hemagglutination dose was previously determined by titration as the smallest amount of virus which would agglutinate 0.1 cc. of 2.5 per cent cells in a final volume of 0.5 cc. Unless otherwise stated, human group O cells and chick embryo allantoic fluid infected with PR8 influenza virus were used in the assays. In the actual test 0.2 ml. of twofold serial dilutions of the extract to be tested was mixed with two minimal hemagglutinating doses of virus contained in 0.2 ml. of saline buffered to pH 7.4 by addition of 10 per cent of 0.15 molar phosphate buffer. The mixture was kept at 4°C. for one-half hour and then 0.1 ml. of a 2.5 per cent suspension of red cells in buffered saline was added, the mixture was shaken, and the cells were allowed to settle in the cold room. The degree of agglutination was read by the pattern of the sedimented red cells after the method of Salk (6). The titer represents the reciprocal of the highest dilution of inhibitor showing complete absence of agglutination.

Extraction of Inhibitor from Red Cells.—The first step was the conversion of cells[1] to stroma.[1] In earlier experiments the inhibitor was extracted from pools containing only group O cells. It is therefore evident that although it has been reported that blood group specific substance A exerts an inhibitory effect on hemagglutination by PR8 virus (5), the inhibitor under consideration is not the A or B antigen. This is further substantiated with respect to the A antigen by the fact that a highly purified specimen of group A substance from hog stomach[2] did not inhibit hemagglutination in a concentration of 1 mg. per cc. It is also of interest that a preparation of hog's stomach group O substance[2] did not inhibit hemagglutination at a concentration of 250 gamma per cc. Friedewald (3) has also reported group A substance to be inactive as an inhibitor of chicken cell agglutination by influenza virus. Owing to the difficulty of securing adequate amounts of blood the later experiments were done with pools of all the types. There was no significant difference in the amount or quality of extractable inhibitor obtained.

In the earlier experiments the cells were washed three times with saline. Since inhibitor material from unwashed cells was the same with respect to yield and behavior as that obtained from washed cells, and since samples of pooled plasma showed no inhibitor extractable by the method outlined below, this tedious step was omitted in later preparations.

The stroma was prepared after the method of Jorpes (7). The packed cells were lysed by the addition of ten volumes of cold (5°C.) distilled or tap water, and the pH was brought to 5.6 by the addition of 1 per cent HCl or acetate buffer pH 4.0 (700 ml. 10 N HAC, 200 ml. 7 M NaAC,

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[1] Cells and stroma were kindly supplied by the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, and by the Sharp and Dohme Company, Glenolden, Pennsylvania.

[2] This was kindly made available to us through the courtesy of Dr. E. A. Kabat, Columbia University, College of Physicians and Surgeons.

400 ml. distilled water). The stroma settled out in a thick curd which was separated in the Sharples centrifuge. The pink precipitate was diluted with distilled water to a volume roughly one-fifth that of the original, packed red cells, and the pH of the thick fluid was brought to 7.1–7.2 by the addition of 0.5 N NaOH with vigorous stirring.

Calvin *et al.* (8) have shown that stroma prepared as described consists of two fractions, elinin and stromatin, separable by high speed centrifugation over a pH range of 7.5 to 7.8. It was of interest to determine which of these two fractions carried the inhibitory activity.

Stroma was suspended in buffered saline pH 7.4 to give a pinkish opalescent solution. On spinning this suspension at 18,000 R.P.M. for 30 minutes, the elinin was found as a pinkish grey pellet on the bottom of the tube. The clear supernatant stromatin solution was pipetted off, the elinin was resuspended in the original volume of buffered saline, and the two solutions were assayed for activity.

The titer of the stromatin solution was less than twenty, while that of the elinin suspension was 2500. Obviously the activity is associated with the elinin fraction.

Various attempts to resolve the elinin particles into soluble components with preservation of activity were unsuccessful until finally the fact that Calvin *et al.* had reported it to be a lipoprotein suggested the use of the McFarlane (9) procedure for dissociating lipoproteins. This consisted of alternate freezing and thawing of an aqueous suspension or solution of the material in the presence of diethyl ether. The procedure when applied to stroma yielded the inhibitor in an ether-soluble form.

Whole stroma paste, pH 7.0, was distributed in 70 ml. amounts into 250 ml. bottles. One and one-half to two volumes of peroxide-free ether were added to each bottle, which was then stoppered and shaken vigorously until an emulsion had formed. The unstoppered bottles were then placed in a dry ice box until the emulsion was frozen. After thawing, the bottles were stoppered, shaken vigorously, centrifuged, and the ether was then siphoned off. Fresh ether was added, and the procedure was repeated six times. On further extraction the yield of ether-soluble active material decreased although the inhibitor content of the stroma was not entirely exhausted. Similar results were obtained in the extraction of lung tissue (Fig. 1).

The combined pale yellow ether solutions were evaporated *in vacuo* under a stream of CO_2 to a light brown mud which was kept under suction until most of the water was removed. This was then thoroughly triturated with acetone (200 ml. per liter of starting stroma suspension) and the supernatant was separated by centrifugation and decantation. The extraction was repeated two or three times until the acetone washings were colorless. The same process was repeated with 95 per cent alcohol, using approximately 75 ml. of alcohol per liter of stroma for each extraction, until the alcohol washings were colorless. It was found that not only was all the activity left in the acetone- and alcohol-insoluble fraction, but that the inhibitory titer was actually increased by the passage into the acetone and alcohol of some material which antagonized the inhibitor (Table I). This antagonistic effect is probably due to the action of some component of the acetone- and alcohol-soluble fraction on the cells, since this fraction, when suspended in saline, caused agglutination of the cells in the absence of virus.

Following the acetone extraction, the residue remained in the form of a light brown dry powder, whereas it is a gummy mass after extraction with alcohol. Therefore it was found

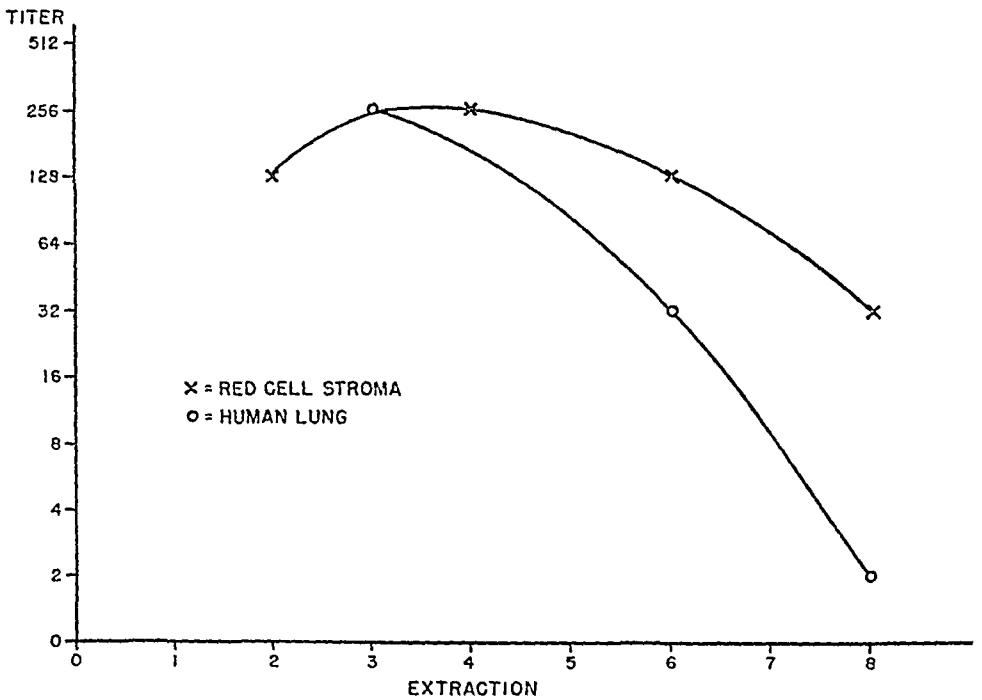

FIG. 1. Titer of successive extractions of human lung and red cells.

TABLE I

Removal of Inhibitor Antagonist by Acetone and Alcohol Extraction

	Dilution of inhibitor								
	2	4	8	16	32	64	128	256	512
A	−	+++	+++	+++	+++	+++	+++	+++	+++
B	−	−	−	−	−	−	−	−	+++
C	−	+++	+++	+++	+++	+++	+++		
D	−	−	−	−	++	+++	+++		
E	−	−	−	−	−	+	+++		

A, batch 14. Residue of 100 ml. ether extract resuspended in 6 ml. saline.

B, batch 14. Residue of 100 ml. ether extract treated with acetone and alcohol and resuspended in 6 ml. saline.

C, batch 28. Residue of 100 ml. ether extract resuspended in 3 ml. saline.

D, batch 28. Residue of 100 ml. ether extract treated with acetone and resuspended in 3 ml. saline.

E, batch 28. Residue of 100 ml. ether extract treated with acetone and alcohol and resuspended in 3 ml. saline.

convenient to treat the alcohol residue once again with acetone to obtain the active insoluble material in the powdered form. Activity at this point was about 0.1 unit per gamma, and the yield averaged 2 to 3 gm. per liter of original stroma. This powder was suspended in chloro-

form overnight, then filtered through a sintered glass filter of medium porosity, washed, and dried. A considerable amount of substance, usually about 50 per cent, dissolved in chloroform to give a dark brown solution, and approximately 15 per cent of the activity was often found in this soluble fraction. The chloroform-insoluble brown powder contained most of the inhibitor and its activity was of the order of 0.16 unit per gamma.

The active material was water-soluble and could be extracted almost completely by allowing the powder to soak in water overnight in the refrigerator, or more rapidly by heating an aqueous suspension of the powder at 60°C. for 20 to 30 minutes in a water bath. On centrifuging the water-insoluble material at 18,000 R.P.M. (25,000 *g*) in the high speed angle head centrifuge for 30 minutes, clear, light brown aqueous solutions were obtained. The activity of these solutions was of the order of 1.25 units per gamma of dry weight. The active solutions gave a positive Molisch test and contained 7 per cent N. The active material was apparently a substance of high molecular weight as it was non-dialyzable. It was sedimentable in the ultracentrifuge at 200,000 to 240,000 *g* but was obviously impure, as the solution showed several components in the analytical ultracentrifuge. About one-quarter of the material appeared to be a polydisperse fraction sedimenting very readily in the ultracentrifuge (sedimentation constants greater than 15 S). Slightly more than half of it sedimented fairly uniformly, with sedimentation constants of 10 to 12 S. The remainder was found in two minor components sedimenting more slowly. The active component was in the more rapidly sedimenting fractions, and probably in the one coming down first. This is only a tentative conclusion as a clear separation of the two most rapidly sedimenting fractions was not achieved. Further purification of the fractions was not attempted since it was found possible to prepare decidedly more active material by another method, as follows:—

Preparation of Chloroform-Soluble Material.—The freezing and thawing of the stroma-ether mixture was cumbersome, and a more convenient method of extraction was desired. This was achieved by heating a neutral stroma paste containing a few per cent of ether at 70°C. for 15 minutes. The mixture was cooled to room temperature afterwards and extracted with approximately 3 to 4 volumes of ether, letting it remain at room temperature for 24 hours with frequent shaking. Occasionally a relatively stable emulsion was formed on shaking, which broke only on addition of more ether, or after prolonged standing. The extraction was repeated three times. Further extractions resulted in decreasing amounts of activity.

After removing the ether by distillation *in vacuo* the residue was dried and treated with acetone and alcohol as previously described. At this point it was found that the activity lay in the insoluble light brown residual powder as before, but that, in contrast with the findings at the corresponding stage in the previous methods of preparation, this powder was almost completely soluble in chloroform. On treating it with this solvent, there remained a slight amount of insoluble brown gum, possessing some activity, which was removed by filtration through a sintered glass funnel. The resulting clear, amber-colored fluid contained about 90 per cent of the activity.

Addition of seven volumes of acetone to the filtrate gave a yellow, flocculent precipitate containing all of the activity. After decanting the supernatant the precipitate was dissolved in a minimal amount of chloroform and one and one-half volumes of acetone was added. A light flocculent precipitate separated out. This contained 80 per cent of the activity. A moderate amount of dark brown impurity remained in the supernatant soluble portion, which was decanted after centrifugation. The precipitate was redissolved in a minimal amount of chloroform and reprecipitated with one and one-half volumes of acetone. It dried to an amber-colored solid which slowly dissolved in water to give a ropy solution. The activity of this solution was approximately 0.3 unit per gamma. It contained lipid, as evidenced by the appearance after a short period of hydrolysis of an acid-insoluble, ether- and alkali-soluble oil. There was very little of this lipid however, and no substance possessing activity could be extracted from the untreated, aqueous solution, on shaking with ether.

The marked difference in chloroform solubility between the inhibitor solution obtained by the first and second of the methods described was of some interest, since it presented the possibility that two different substances were involved. However, it was found that the chloroform-soluble inhibitor could be converted into the chloroform-insoluble form by freezing and thawing an aqueous solution in the presence of ether and a high partial pressure of carbon dioxide. Without this last the conversion was not effected. It was also found that ether extraction of the thoroughly dried material for 5 or 6 days at room temperature was partially effective in converting the powder to the chloroform-insoluble form. It seems likely that there was a lipid complex involved, the dissociation of which was subject to a pH effect. This possibility was supported by one experiment in which it was found that conversion of the chloroform-soluble active material to the chloroform-insoluble form had occurred after evaporation of a small amount of the former to dryness by means of a stream of CO_2. The point, however, has not been thoroughly investigated.

Conversion of the Chloroform-Soluble to the Insoluble Form.—A 0.390 gm. sample of chloroform-soluble powder containing 125,000 units of activity was dissolved in 10 ml. of water and shaken with an approximately equal volume of ether. No color went into the ether solution. The mixture was frozen and thawed under the conditions noted above. On shaking after each thaw the ether layer was seen to become increasingly colored. Following the sixth thaw the ether was siphoned off and the water layer was repeatedly extracted with portions of ether until no more color went into the ether layer. At this point the water layer was faintly straw-colored. The water was removed by lyophilization, the residue was extracted with ether, chloroform, and petroleum ether, and dried, providing an almost white powder. On resuspending in 4 ml. of water, this powder went slowly into solution after swelling into jelly-like particles. A small amount of water-insoluble material was separated by centrifugation. The clear viscous supernatant had a solid content of 12.0 mg. per ml., and had an activity of 2.5 units per gamma against both mumps and PR8 viruses.

The nitrogen content of this material, determined by a micro modification of the Nessler method (10) was 2.6 per cent. It gave a positive ninhydrin test. There was no inactivation on prolonged shaking with chloroform. A quantitative determination showed no glucosamine to be present.[3] Further information on the nature of the nitrogenous component will await the preparation of more material. There was no detectable phosphorous present in a 100 gamma sample.

The material contained carbohydrate as evidenced by the presence of a positive Molisch test. The quantitative orcinol test for pentoses (11) and the Dische test (12) for uronic acids were completely negative when assayed on 100 gamma samples. Under the conditions used 10 gamma control samples of xylose and Type III pneumococcus polysaccharide showed definite color. The unhydrolyzed material gave a reducing value of 1 per cent (as glucose) by the Malmros modification of the Folin-Wu method for glucose (13). On hydrolysis

[3] This determination was kindly performed by Mr. Cava through the courtesy of Dr. Karl Meyer of Columbia University.

the reducing sugar, determined as glucose equivalents, was found to be 48 per cent. The maximum reducing value on hydrolysis with 2 N HCl was found to occur in 1 hour, after which time it slowly decreased. The inhibitor is extremely labile to acid hydrolysis, being completely inactivated by treatment with 0.1 N HCl for 5 minutes and 75 per cent inactivated by 0.001 N HCl for 10 minutes in a boiling water bath. One-thousandth normal NaOH at 100°C. for 10 minutes caused no loss of activity. A slight amount of insoluble material appeared on acid hydrolysis but the nature of this has not yet been investigated.

A 1 per cent solution showed no measurable optical activity in a 1 dm. tube.

This preparation was completely sedimentable in the analytical ultracentrifuge but showed no sharp peak, behaving as a polydisperse material with a minimum sedimentation constant of about 20. This bears out the previously described results obtained with material in which it was indicated (but not clearly demonstrated) that the active component of this impure preparation was in the most rapidly sedimentable fraction.

These purified inhibitor solutions were readily inactivated by virus. In one experiment, 12,000 units of inhibitor were inactivated by 300 hemagglutinating units of PR8 virus in 16 hours at room temperature with recovery of 30 per cent of the activity of the virus. In the absence of the virus the inhibitor proved stable in watery solution at room temperature.

DISCUSSION

From the above data it is probable that the water-soluble, chloroform-insoluble inhibitor is polysaccharide in nature. This is not surprising, especially when considered in connection with the previously mentioned findings of Horsfall, Woolley, and the still earlier reports of the inhibitory effects of polysaccharides on bacteriophage (14) and on plant viruses (15). Whether or not it is a complex protein or lipopolysaccharide it is impossible to say until further information is available on the state of purity of the active inhibitor and the chemical nature of its structural components. It is evident that the chloroform-soluble form of the inhibitor is associated with the presence of lipid material. The conditions under which the lipid can be separated from the chloroform-soluble active material indicate the possibility of a complex consisting of lipid and water-soluble, chloroform-insoluble inhibitor. There are, of course, many known types of lipoprotein and lipopolysaccharide complexes, and the nature and stability of the binding forces of these complexes show wide variation. It is possible that the active ether- and chloroform-soluble substance represents material that is less degraded than the water-soluble inhibitor, and it might, therefore, be a closer approximation to the natural state of the receptor as it exists in the red cell. On the other hand, the possibility exists that the association of lipid and inhibitor, responsible for the chloroform solubility of the latter, is merely an artifact caused by the method of preparation. Further in-

vestigations should reveal more precisely the nature and characteristics of the material under consideration.

It is of interest that our inhibitor is more active in preventing hemagglutination than any of the numerous polysaccharides from various sources and the other materials tried by Woolley. His most active preparations gave almost complete inhibition of agglutination in concentrations of approximately 100 gamma per ml. In a roughly comparable test our most active material gave complete inhibition, that is "O or trace" of agglutination by Woolley's criteria, in a concentration of 1.1 gamma per ml.

Friedewald and collaborators also described the preparation of inhibitor in high titer by a saline extraction of human red cells. In some experiments on specificity of inhibition, and elution of virus from inhibitor, they obtained data similar to our results already described (1). They also arrived at the conclusion that the active material in the extracts represented the receptor substance of the red cell. Their active extracts were prepared by lysing red cells in a Waring blendor followed by centrifugation of the mixture at 3,000 R.P.M. It would be of interest to know whether these extracts represent a soluble inhibitor or a suspension of finely divided stroma (elinin). In our experience elinin prepared by hemolysis of red cells remained suspended, with retention of inhibitor activity, after centrifugation at 3,000 R.P.M. at pH 7.0; but it was sedimented at 18,000 R.P.M. for 20 minutes to the extent of 90 per cent as estimated by the disappearance of inhibitor activity in the supernatant.

Friedewald and collaborators describe the preparation of active extracts from various animal and human tissues, and raise the question as to the identity of tissue and red cell inhibitor. In this connection it is of interest that although we have been able to obtain from human lung an ether-soluble inhibitor, we have not been able to find similar material in human liver, kidney, or serum. This and various other biological properties of the inhibitor substances are under investigation.

SUMMARY

Methods have been described for the extraction and purification of an agent inhibiting the hemagglutination of red cells by influenza (PR8) and mumps viruses. Human red cells have served as the chief source of the inhibitor but the latter has also been found in human lung.

The active extracts have been purified to the extent that 0.1 gamma of material suffices to inhibit one hemagglutinating dose of virus. Incomplete chemical characterization of the most highly purified fractions available indicates the presence of 2.6 per cent nitrogen, at least 50 per cent of polysaccharide, and no phosphorus. In the ultracentrifuge the purified preparation behaves as a polydisperse macromolecular substance.

The active material can be obtained from red cell stroma in an ether- and

chloroform-soluble form which, on further treatment, can be converted into chloroform-insoluble material. It is possible that the former represents more closely the virus receptor as it exists in the red cell.

The purified inhibitor is inactivated on incubation with the virus at 37°C. The nature of this effect is being investigated.

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STUDIES ON ENDEMIC PNEUMONIA OF THE ALBINO RAT

III. Carriage of the Virus-Like Agent by Young Rats and in Relation to Susceptibility

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The presence of a virus-like agent in the lung of adult albino rats naturally infected with endemic pneumonia has already been reported from this laboratory (1, 2). The activity of the agent was first demonstrated experimentally by nasal transfer to mice, in which an inflammation of the respiratory tract and middle ears was produced. In attempting to reestablish the agent in the rat certain difficulties were met which led to the study, here presented, of its carriage by the rat at different age levels.

Materials and Methods

Albino rats from the selected colony maintained at the Rockefeller Institute in Princeton were used exclusively in this work (3). These rats are free from infectious catarrh and pleuropneumonia but show a high rate of endemic pneumonia as they reach maturity. The mice were also from a Princeton colony in which there is practically no respiratory disease. Young animals 4 to 5 weeks old, weighing 15 to 18 gm., were employed. Their age was not a significant factor, earlier work having indicated that immature mice were as susceptible as adults. Lung suspensions from both animals were prepared in a concentration of approximately 10 per cent in saline or buffered saline using a glass tissue grinder. All nasal injections were made with a syringe in animals previously anesthetized with ether. The volume of the inoculum was generally 0.05 cc. in mice and 0.1 cc. in rats. At the end of the observation period the mice were killed with ether and decapitated. At autopsy the lung was removed aseptically in a Petri dish and examined under a low power dissecting microscope for areas of consolidation. The middle ears and nasal passages were opened and inspected in all rats and in mice which showed no pneumonia. Unless otherwise stated the interval between nasal injection and autopsy was 4 weeks.

Transmission Experiments in the Rat

Young rats were used in the transmission tests, the choice being based in part on an assumed susceptibility and in part on the low natural rate of pneumonia. In our colony approximately 10 per cent of the immature animals show pneumonic foci in the lung at autopsy. However, the initial experiments in which pneumonic lung suspensions from adult rats were employed indicated a refractory tendency on the part of many young rats. As shown in Table I, the incidence of pneumonia in 2 to 3 months old rats 4 weeks after nasal injection was not significantly higher than in uninjected rats of the same age, 12 as com-

pared with 10 per cent (1). In contrast, the observed incidence in mice injected with the same suspensions and killed 4 weeks later was 100 per cent.

It was largely because of the refractory state of young rats that white mice were chosen as alternative hosts. Having shown that pneumonic lung suspensions from adult rats regularly produced a disease of the respiratory tract in mice, it became particularly important to reattempt the establishment of the virus-like agent in the rat as bearing on its causal relation to endemic pneumonia. There was a possibility, moreover, that lung suspensions from the mouse would prove more favorable than rat suspensions by reason of enhanced virulence induced by passage.

Accordingly a second series of transmission tests was begun in the rat, using lung suspensions from experimentally infected mice. The results of these tests employing 2, 3, and 6 months old rats are summarized in Table II. The

TABLE I

Results of the Nasal Injection of Young Rats and Mice with Pneumonic Lung Suspensions from Adult Rats

No. of adult rat	Age of adult rat	No. of young rats injected	No. with pneumonia at autopsy	No. of mice injected	No. with pneumonia at autopsy
	mos.				
1	14	5	0	3	3
2	14	5	0	3	3
3	13	5	2	5	5
4	12	5	1	5	5
5	14	5	0	5	5

five rats used in each experiment were usually litter mates which had been kept together in the same cage since they were weaned. The initial injections were made in 2 months old rats which were killed and autopsied 4 weeks later. The pneumonia rate being low, the interval between injection and autopsy was subsequently lengthened to 2 months but with no significant change in the findings. The over-all incidence of pneumonia for the 25 rats in this age group, which were held for a period of 1 to 2 months, was 16 per cent.

An identical rate was obtained for the 25 rats which were 3 months old at the time of injection. Here again, increasing the interval between injection and autopsy had no appreciable effect. The rats in the third age group, which were 6 months old when injected, and then were held for 2 to 3 months, showed a marked increase in the incidence of pneumonia, the over-all rate being 84 per cent. All of the suspensions used in these experiments were simultaneously tested in mice and were uniformly infective.

One additional transmission experiment was made with two groups of five recently weaned rats, approximately 3 weeks old. They were killed 4 weeks

after the nasal injection of an active mouse lung suspension and showed no pneumonia at autopsy.

It was evident from the combined results of the transmission tests that young rats throughout the 3rd month were essentially as refractory to the virus-like agent passaged in mice as they were to that recovered directly from rats. The high rate of pneumonia observed in the 6 months old rats can hardly be regarded as evidence that the injected agent was the initiating factor, as a correspondingly high rate may occur in the same number of uninjected rats of the same age.

TABLE II

Results of the Nasal Injection of Rats with Pneumonic Lung Suspensions from Mice

No. of experiment	Age of rat when injected	No. of rats injected	Interval between injection and autopsy	No. of rats with pneumonia at autopsy
	mos.		*mos.*	
		5	1	1
		5	1	0
1	2	5	1	0
		5	2	1
		5	2	2
		5	1	1
		5	2	0
2	3	5	2	2
		5	2	0
		5	2	1
		4	2	4
		5	2	2
3	6	5	2	5
		5	3	5
		6	3	5

Detection of the Virus-Like Agent in Young Rats Following Nasal Instillation

In attempting to account for the insusceptibility of the young rat attention was first directed towards the fate of the experimentally introduced agent. The lungs from the two groups of recently weaned rats above mentioned, ten in all, which were uniformly normal at autopsy, presented suitable material for this determination. A composite suspension made from the lungs of each group of five was injected intranasally in mice. All of the injected mice showed pneumonia at autopsy, indicating that a detectable concentration of active virus-like agent was present in the seemingly normal rat lung through the 4th week after nasal instillation.

Carriage of the Virus-Like Agent by Uninjected Rats

The preceding experiment having indicated that the virus-like agent was detectable in the lung of young rats after injection, it was essential to determine whether it was acquired as a result of this procedure or existed as a result of natural transmission. It was looked for in the lungs of normal rats removed directly from the breeding colony. The first test was made with three recently weaned rats which apparently were not harboring the agent. A composite lung suspension from the three rats did not produce pneumonia on nasal transfer to mice. A subsequent experiment with five 2 weeks old unweaned rats likewise failed to show the presence of the agent in the lung. Three later tests with 4 weeks old rats proved conclusively, however, that the young rat was a potential carrier of the virus-like agent. Composite lung suspensions from each of the three groups of five rats uniformly produced pneumonia on transfer to mice.

The real clue to the situation which existed in the breeding colony finally came through the injection of nasal washings from young rats. In all of the preceding experiments involving immature animals attention had been paid to the nasal passages and middle ears, in addition to the lungs, with normal findings; there was no exudate in the nasal passages and the small amount of material removed by aspiration showed no polymorphonuclear leucocytes microscopically. Nasal washings from the 2 weeks old rats which failed to show the virus-like agent in the lung did, however, produce pneumonia on transfer to mice. It was thus indicated that the agent was present in the upper respiratory tract at an early age and the findings suggested that it was acquired by parental contact.

A series of detailed experiments was subsequently made to determine the frequency of carriage by young rats at different age levels and the means by which the virus-like agent was conveyed to them.

In these experiments the offspring of female rats from the selected breeding colony were used. The latter were young breeders, 6 to 7 months old, and in their first or second pregnancy. The environmental conditions under which the young rats were reared simulated the natural state of affairs in the breeding colony. The only real departure was the isolation of the mother rat, either prior to parturition or not later than 24 hours thereafter. Ordinarily the young are born in the breeding cage which houses four females and one male and thus are exposed to a group of adult animals until weaned.

Each litter of newborn rats was divided into two groups which were tested at different intervals, one group during the suckling period and the other subsequent to it. Most of the young rats in the second group were left in contact with their mothers until they were killed.

In the earlier work with endemic pneumonia the diagnosis of nasal inflammation was based on the demonstration of a definite mucopurulent exudate by aspiration with a capillary pipette. Microscopic examination of nasal washings was not carried out in the absence of exudate. In the present series the nasal passages of both young and adult rats were washed with a small amoung of saline, usually 0.5 cc., and the resulting washings examined microscopically after Gram staining.

The entire lung from each rat, save for several small pieces removed for sectioning, was

ground and suspended in sufficient saline solution to make a concentration of about 10 per cent. The lung and nasal suspensions were each injected intranasally in three or five mice. These injections were made within 2 hours of the time the suspensions were prepared.

The six female breeders examined in these experiments varied in age from 7 to 10 months when killed. At autopsy all of them showed pneumonic foci in the lung. The middle ears were normal. A copious nasal exudate was present in only one rat, the other five rats yielding small amounts of rather thick material which ordinarily would not be considered exudative in nature. Many polymorphonuclear leucocytes were present, however, in stained films of nasal washings from each of the six rats. Intracellular granules suggestive of coccobacilliform bodies or pleuropneumonia-like organisms were absent.

It is probable that our examination of the lung is somewhat more searching than that commonly made. Only one of the six rats showed a lung lesion large enough to be clearly definable macroscopically. In the other five rats the diagnosis of pneumonia was based on the presence of minute discrete areas, rounded and gray, on the surface of the lung. They were barely visible to the unaided eye, if few in number, and were best seen with the aid of a dissecting microscope by which means they were unmistakable. These areas varied in number and distribution from rat to rat and from lobe to lobe but if sufficiently numerous gave the lung a characteristic spotted appearance.

Presence of the virus-like agent in the lung and nasal passages of the six mother rats was indicated by the invariable production of pneumonia on nasal transfer of the respective suspensions to mice. The data obtained from the offspring of the six breeders naturally infected with endemic pneumonia are presented in Table III. The virus-like agent was demonstrable in the respiratory tract of individual rats from all of the six litters.

Five rats killed within 1 day of birth failed to yield the agent from either the lung or the nasal passages, but it was detectable at both situations in the corresponding litter mates killed after weaning, save in one instance in which it was present only in the nasal passages. The specific agent was first demonstrable in very young rats on the 5th day after birth, being recovered from the nasal passages of a single animal out of three tested. After 7 days it was recovered from the nasal passages but not the lung of three of the six rats examined. In both these age groups the agent was later detectable in the lung of the corresponding litter mates. It was recovered from the nasal passages of each of three rats killed on the 19th day, just prior to weaning, and from the lung of two. The three litter mates killed after weaning harbored the agent in both the lung and the nasal passages.

None of the 33 young rats included in the six litters showed either macroscopic or microscopic evidence of pneumonia at autopsy. Inflammation of the upper respiratory tract, indicated by the presence of many polymorphonuclear leucocytes in the nasal washings, was observed in three rats from different litters killed after weaning. The middle ears, examined only in rats killed immediately before or after weaning, were uniformly normal.

The experiment indicated that the virus-like agent was acquired by the albino rat at an early age and that it was introduced by way of the upper respiratory tract. The specific agent was demonstrable in the nasal passages as

early as the 5th day of life but was only later recoverable from the lung. Advantage was taken of this observation in a supplementary experiment in which only nasal washings were used from rats killed within 1 day of birth, and only lung suspensions from their litter mates killed after weaning.

TABLE III

The Findings in Young Rats at Different Age Levels

No. of litter	Group 1				Group 2			
	No. of rat	Interval between birth and autopsy	Transfer of nasal washings to mice	Transfer of lung suspension to mice	No. of rat	Interval between birth and autopsy	Transfer of nasal washings to mice	Transfer of lung suspension to mice
		days				*days*		
1	1	1	−*	−	4	33	−	+
	2		−	−	5		+	+
	3		−	−				
2	1	1	−	−	3	35	+	+
	2		−	−	4		+	+
3	1	5	+	−	4	34	+	−
	2		−	−	5		+	+
	3		−	−	6		+	+
4	1	7	−	−	4	33	+	+
	2		−	−	5		+	+
	3		−	−	6			
5	1	7	+	−	4	42	+	+
	2		+	−	5		+	+
	3		+	−	6		+	+
6	1	19	+	+	4	41	+	+
	2		+	+	5		+	+
	3		+	−	6		+	+

* In Tables III, IV, and V, + indicates involvement of the lung and/or the middle ears of the injected mice; − indicates that the mice were normal.

Three female breeders, 6, 8, and 6 months old respectively when their litters were born, were used in this experiment. Each of them showed pneumonic foci of the minute discrete type in the lung at autopsy and many polymorphonuclear leucocytes in the nasal washings. Their middle ears were normal. The results of the examination of their young are given in Table IV. The virus-like agent was not recovered from the nasal passages of the 14 young rats killed within a day of their birth. It was demonstrable, however, in the lung of seven of the ten corresponding litter mates killed after weaning. The lungs, middle ears and nasal passages of these rats were uniformly normal at autopsy.

One additional transmission test is included since it illustrates the variability which may occur in litters tested shortly after the weaning date. In this experiment a 6 months old female breeder and her six young were isolated within a day of parturition. Twenty-five days later the mother and all of her offspring were killed. The results of the transmission test in which five mice were injected with each suspension are given in Table V. The virus-like agent was present in both the nasal passages and lungs of three of the young rats. It was present only in the nasal passages of one rat and only in the lung of another.

TABLE IV

The Findings on Partial Examination of Young Rats at Different Age Levels

No. of litter	Group 1			Group 2		
	No. of rats used	Interval between birth and autopsy	Transfer of nasal washings to mice	No. of rats used	Interval between birth and autopsy	Transfer of lung suspension to mice
		day			*days*	
7	6	1	6 −	2	35	2 +
8	3	1	3 −	3	21	2 +
9	5	1	5 −	5	21	3 +

TABLE V

Results of the Examination of Recently Weaned Rats and Their Mother

No. of animal	Transfer of nasal washings to mice	Transfer of lung suspension to mice
Adult rat 10	+	+
Young rat 1	+	−
" " 2	+	+
" " 3	+	+
" " 4	+	+
" " 5	−	+
" " 6	−	−

It was not demonstrable in either the nasal passages or the lung of one animal the first uninfected rat observed of the 38 weaned young which were fully tested.

Titer of the Virus-Like Agent in the Lungs of Young and Adult Rats

Having shown that the virus-like agent was recoverable from the lungs of adult rats in the presence of pneumonic lesions and from young rats in the absence of any apparent pulmonary alteration, it was important to determine the titers in animals of the two sorts. Titration of the agent in young rats was subject to the chance utilization of an infected animal as there was no way of

determining beforehand whether or not infection had occurred. As a matter of record the first test was made with a suspension from a 3 weeks old rat, the only one of a group of three which failed to show the agent in the lung.

In these tests the entire lung was removed aseptically, weighed, and after suspending in saline solution with a glass tissue grinder, diluted to a concentration of 5 per cent. Subsequent tenfold dilutions were then made with saline. The volume of inoculum was 0.05 cc. which was included in the final dilution figure. Five mice were injected intranasally with each dilution ranging from 10^{-2} through 10^{-7}. They were killed a month later.

The maximum dilutions of the normal lung tissue of two young rats which produced pneumonia or otitis media on intranasal injection in mice were 10^{-4} and 10^{-5} respectively. Both of these rats were 3 weeks old when killed and were from two different litters. The titers of the agent in the pneumonic lungs of two adult rats, 12 and 14 months old, were 10^{-6} and 10^{-5} respectively.

DISCUSSION

The detailed examination of entire litters and the female rats which had reared them gave evidence that the virus-like agent was readily transmissible by direct contact and was probably passed from mother to young by way of the upper air passages during the suckling period. In spite of the high rate of infection in young rats comparatively few of them showed any pulmonary or nasal lesions at autopsy. The virus-like agent recovered from these animals in the absence of lesions was fully as active on transfer to mice as that from adult rats with pneumonia and rhinitis. The limited titrations that were made indicated that the amount of agent present was somewhat less in the immature animals. The difference in titer was not sufficiently great, however, to account for the inactivity of the agent in the young rat. It seems probable that some factor is operative in the young rat during its growth period which checks the activity of the agent without greatly retarding its multiplication. It is possible, though not proved, that the young rat acquires a specific protective element by direct or indirect maternal transfer.

The equilibrium which is apparently established between the rat and the virus-like agent is such that it assures the perpetuation of both, exemplifying a relationship discussed in detail by Theobald Smith (4) and by Burnet (5). The agent is acquired by the rat shortly after birth and thereafter is evidently harbored in the respiratory tract throughout the entire life span of the host. If the agent is procured from the rat and transferred to the mouse it produces a well defined reaction within a period of several weeks and may cause death. In the rat, however, the agent commonly resides in the lung and nasal passages for a period of months without producing a detectable pathologic change. Even in adult rats the reaction associated with it is generally of a low order and so well tolerated that it is usually recognized only at autopsy. The slight damage

done by parasite to host is matched by the inability of the latter to rid itself of the parasite. The long continued residence of the agent in the rat affords ample opportunity for its maintenance and spread within the colony. Its availability for dissemination is shown by the present work and intimated by the seemingly universal distribution of pneumonia in the albino rat.

The prevalence of natural infection offers an explanation for the failure to establish the virus-like agent in young rats by experimental means. It would seem that the experiments had the effect merely of introducing an additional increment of the agent which the young rat was prepared to hold in check. The situation encountered in the selected colony reemphasizes the importance of disease-free populations for the study of infectious disease in general and of rat pneumonia in particular.

SUMMARY

An attempt was made to reestablish the virus-like agent associated with rat pneumonia by the nasal instillation of the infectious material into supposedly normal young rats from the selected Princeton colony. The incidence of the pneumonia in these animals after the injection of suspensions of pneumonic lungs from either rats or mice was not significantly greater than the incidence under natural conditions.

In attempting to account for the refractory state of the immature rats it was found that the agent was widely dispersed through the breeding colony at an early age. Detailed tests of many litters indicated that the agent was acquired shortly after birth by way of the upper air passages as the result of maternal contact. In most instances it was so well tolerated by young rats during the suckling period and for several months thereafter that its presence was recognized only by nasal transfer to mice.

There is every indication that the rate of infection of rats in the breeding colony approaches 100 per cent by the time the animals are old enough to be used experimentally.

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STUDIES OF THE DISTRIBUTION OF POLIOMYELITIS VIRUS

V. The Virus in Familial Associates of Cases*

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During the past decade much attention has been directed toward the recovery of poliomyelitis virus from the intestinal tract of individuals afflicted with the disease. In addition, various workers have reported the isolation of virus from apparently healthy persons who were associated, in different degrees of intimacy, with actual cases (1–14). The results of these studies are presented in Table I where it will be seen that of 494 so called contacts, 116, or 23 per cent were excreting virus in their intestinal discharges *at the time of testing*. It must be emphasized that most of the above attempts at isolation were made from single specimens obtained from the individuals several days to several weeks following exposure to a case. The number of individuals positive for virus might have been greater because stool specimens in three of the studies were pooled for testing and more than one individual in some pools might have been carrying virus. Some of the individuals whose stools contained virus suffered minor illnesses which may have been abortive attacks of the disease. Of the associates studied, 277 were identified as either children or adults. Among the children, 69 of 228 (30 per cent) were positive and 8 of 49 (16 per cent) of the adults harbored virus in their intestinal contents.

Lépine and his coworkers (15) reported the successful isolation of virus from the child of a father afflicted with the disease, 41, 74, and 123 days after a mild affection which may have been abortive poliomyelitis. Ward and Sabin (7) found virus in the stool of one sibling at 4 weeks but not at 9 and 13 weeks after a brother's illness; and in the stool of another sibling at 4 and 9 weeks but not at 12 weeks. They considered the possibility that "these siblings might have been the carriers which served as sources of infection." Wenner and Casey (16) reported positive stools collected from a child 2 and 6 weeks following a poorly defined illness but not after 11 weeks. In studies of familial associates of frank cases in Fort Worth, Texas, single specimens were found positive as late as 7 weeks after the onset of the diagnosed cases (10).

The above examples are representative of the few studies in which repeated sampling of associates has been attempted. If the rôle of the familial associate

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as a healthy carrier or a possible source of infection is to be evaluated specimens should be obtained at frequent and regular intervals beginning as soon as possible from the onset of illness in the family. The present report describes the results of such a study.

TABLE I

Recovery of Poliomyelitis Virus from Stools of Associates

	Number positive	Numbernegative
1. Kramer, Gilliam, and Molner (1939)	6	17
2. Trask, Paul, and Vignec (1940)	0	36
3. Kessel, Moore, Stimpert, and Fisk (1941)	1	18
4. Piszczek, Shaughnessy, Zichis, and Levinson (1941)	8	32
5. McClure and Langmuir (1942)	20	7
6. Howitt, Buss, and Shaffrath (1942)	6	25
7. Ward and Sabin (1944)	2	6
8. Brown, Francis, and Pearson (1945)	5	1
9. Gear, Yeo, and Mundel (1945)	2	5
10. Pearson, Brown, Rendtorff, Ridenour, and Francis (1945)	14*	93
11. Melnick, Horstmann, and Ward (1946)	3*	72
12. Gear and Mundel (1946)	7	9
13. Gordon, Schabel, Casey, Fishbein, and Abendroth (1947)	26	22
14. Pearson, Brown, and Rendtorff (unpublished)	16*	35
Total	116	378
Positive, *per cent*	23	

* Specimens pooled—may be greater number positive.

Collection of Materials

Four families residing in Detroit or its environs were selected for study in Sept., 1946. Two criteria were required: first, that the family include at least two children other than the case; and second, that collections could be started within 3 days of onset of illness in a member of the family.[1]

Family I consisted of the parents, both 38 years old, and three children, 3, 7, and 12 years. There was nothing unusual in the activity of the family except for a motor trip through Canada to Connecticut and back, from July 27 to Aug. 11. The child of 7 developed headache and fever on Sept. 4, was diagnosed as having bulbar poliomyelitis on admission to the hospital the next day, and died Sept. 7. It is of interest that an abscessed molar tooth had been extracted 5 days prior to onset. The youngest brother, 3, had been feverish on Sept. 2; otherwise the family had been and were, in good health.

[1] The original information concerning the date of onset of symptoms and diagnosis was made available through the kind assistance of Dr. Franklin Top, Medical Director, and Dr. C. G. Jennings, both of Herman Kiefer Hospital, Detroit, Michigan.

Pharyngeal washings of gargled distilled water from the parents and the oldest boy and pharyngeal swabs from the younger child were obtained on Sept. 6, 9, 13, 20, 27, Oct. 4 and 25, and Nov. 29.[2]

Stool specimens were collected Sept. 8, 13, 20, 27, Oct. 4 and 25, and Nov. 29. No recognizable illness occurred in this family during the period of study.

Family II consisted of the parents, 39 and 36 years old, and four children, 15, 12, 11, and 6 years. The boy, 11, had headache and sore neck but no fever on Sept. 11. The next day nausea and vomiting occurred and on Sept. 13 typical signs of mild bulbar poliomyelitis were noted. He remained at the hospital during the time of the study. The father had a cold and headache on Sept. 11 as did the brother, 15, 3 days later. A sister, 6, had a cold and "sore stomach" on Oct. 4, 20 days after this last incident. No other illness was observed during the period of observation.

Garglings were obtained on Sept. 14, 16, 20, 27, Oct. 4, 11, 25, and Nov. 29. Stools were collected Sept. 16, 20, 27, Oct. 4, 11, 25, and Nov. 29.

Family III consisted of parents, 30 and 27 years, children 8 and 6 years, and their uncle, 22. The father, 30, complained of headache, nausea and vomiting, and had an oral temperature of 101° on Sept. 13. He was hospitalized on Sept. 15 at which time both legs were paralyzed. Ascending paralysis involving the diaphragm, intercostal muscles, and right arm necessitated his being placed in a respirator on Sept. 17 where he remained throughout the period of study. The only other illness observed in the family was a severe headache and nausea experienced by the wife, Oct. 11.

Pharyngeal washings were obtained Sept. 16, 18, 20, 27, Oct. 4, 11, 25, and Nov. 29. Stool specimens were dated Sept. 16–17, 20, 27–28, Oct. 4, 11, 25, and Nov. 29

Family IV consisted of parents, 45 and 44 years, and children 14 and 11. The father, 45, experienced nausea and vomiting on Sept. 12, difficulty in swallowing the next day, and on Sept. 14 a change in voice and other signs of bulbar poliomyelitis necessitated hospitalization where the illness progressed rapidly to death on Sept. 15. The daughter, 14, had complained of nausea and diarrhea on Sept. 10, but seemed perfectly well the next day and her symptoms were attributed to the onset of menses. Nausea recurred with vomiting on Sept. 14 and she was confined to bed with symptoms described as "psychological" as a result of extreme affection for her deceased father. During this period she complained of not being able to walk although her physician could detect no muscular weakness. When this condition persisted, she was admitted to the hospital for examination on Oct. 2 at which time spasms of the hamstrings and left side of back, and weakness of left quadriceps and anterior tibialis were demonstrated. She remained in the hospital for 2 weeks and when discharged was emotionally better, even cheerful and happy, and without paralysis.

The mother and the other child, a boy aged 11, had been well and remained so throughout the period of study.

Throat washings and stools were obtained on Sept. 16–18, 20, 27, Oct. 4–5, 11–13, 24–27, and Nov. 29.

Testing of Materials

All specimens were preserved in refrigerators with solid CO_2 until tested. Individual stool specimens were thawed, a portion ground with alundum, and suspended in physiological salt solution to approximately 10 per cent by weight. The material was agitated daily for 30 minutes on a shaking machine with

[2] The results of virus studies on throat washings are not complete and will be published at a later date. Tests with specimens taken at the time of the first stool collection from all subjects have, however, been completed and the results have been uniformly negative.

20 per cent ether for 6 to 7 days, after which it was centrifuged at 3000 R.P.M. for 30 minutes in a horizontal centrifuge. The ether was removed by evaporation under low pressure, and the specimen was again centrifuged at 4500 R.P.M. for 30 minutes. Both aerobic and anaerobic sterility tests were applied to each preparation and if no bacterial growth was observed the specimen was inoculated into a *rhesus* monkey. An initial intracerebral injection of 0.5 cc. into the vicinity of the thalamus was followed every 2 or 3 days by inoculations of 10 to 15 cc. intraperitoneally and 2 cc. intranasally until the specimen was exhausted. Daily temperatures were recorded and the animals observed for symptoms a maximum of 30 days. All monkeys which developed paralysis were subjected to autopsy promptly and autopsy was performed at the end of 30 days on animals which had shown any suspicious signs such as elevated temperature, irritability, or ruffled fur. A diagnosis of poliomyelitis was made if sections of nervous tissue revealed typical changes including perivascular cuffing, leucocytic infiltration, neuronolysis, and neuronophagia.

RESULTS

The results of this study are presented in Table II. It will be seen that virus was isolated from the stools of seven of the nine children but from none of the seven adults, among the familial associates. The virus was present in the stools of five at the time of the first collection and in the remaining two 4 days later; in all instances within 9 days of onset of recognized poliomyelitis in a member of the family. There was no consistent relationship between a history of illness and the presence of virus. In only one was illness clinically suggestive of poliomyelitis. Hence, the majority of those positive can be considered to represent inapparent or carrier infections. In three individuals (C.M., A.T., and M.A.), the presence of virus in the intestinal tract was detected on each of five successive collections, covering periods of 25, 26, and 36 days, respectively. Stools from an associate (K.M. in family I) yielded virus on the first, fourth, and fifth collections but were negative during both the 2nd and 3rd weeks; second preparations were made from the negative stools and tested in new monkeys with the same results. This irregularity in the detection of the carrier state was observed in a member of family III (R.S.) whose stools were positive on the first and third collections but negative on the second. It is of interest that this second or negative specimen was collected only 3 days after the first, positive stool. Again, these specimens were retested by processing the original stools and inoculating into new monkeys, with identical results.

The stool of an associate in family II (R.T.) was negative on the first collection but one collected 4 days later was positive. This was followed by four negative specimens.

The seventh individual shown to be positive (N.A.) was carrying virus at

the time of the first and second collections but four successive specimens thereafter were negative. This girl was probably an actual case of poliomyelitis but it is of interest that the muscle weakness was observed *following* the two positive collections, and her intestinal excreta were negative for virus at the time of hospitalization.

TABLE II

Detection of Poliomyelitis Virus in Stools of Familial Associates

Family	Age	Sept.					Oct.			Nov.
		8	13	16–17	20	27–28	4–5	11–13	25–27	29
	yrs.									
I. R. M. (case)	7	(onset Sept. 4)								
F. M.	38	○	○		○	○	○		○	
M. M.	38	○	○		○	○	○		○	
K. M.	12	●	○, ○		○, ○	●	●		○	○
C. M.	3	●, ●	●		●	●	●		○	○
II. J. T. (case)	11	(onset Sept. 11)								
A. T.	39			○	○	○	○	○	○	
M. T.	36			○	○	○	○	○	○	
R. T.	15			○, ○	●, ○	○	○	○	○	
J. T.	12			○	○	○	○	○	○	
A. T.	6			●	●, ●	●	●, ●	●	○	○
III. C. S. (case)	30	(onset Sept. 13)								
F. S.	27			○	○	○	○	○	○	
E. S.	22			○	○	○			○	
R. S.	8			○, ●	○, ○	●, ○	○	○	○	
L. S.	6			○	○	○	○	○	○	
IV. C. A. (case)	45	(onset Sept. 13)								
E. A.	44			○		○	○	○	○	
N. A.	14			●	●	○	○	○	○	
M. A.	11				●	●	●	●	●	○

When two results are presented they represent separate tests with the original specimen.
○ = negative
● = positive

DISCUSSION

The presence of virus in a large percentage of healthy familial associates confirms observations published elsewhere. The persistence of this carrier state, however, has not been thoroughly demonstrated nor has the regularity with which virus may be isolated from successive weekly stool specimens. Three individuals were excreting virus consistently over a period of approximately a month following the occurrence of poliomyelitis in the family and a

fourth was shown to be positive for the same length of time except for a period of at least one week when virus was not recovered. The fact that some carriers may apparently lose that capacity only to regain it is verified by the results with another individual who was found positive on the first and third collections but negative on the second and from the fourth till the end of the study. This irregularity in the excretion of virus by carriers illustrates the difficulty of establishing the percentage of healthy individuals positive for virus when observations are based on single specimens. It may also explain certain differences in percentages reported by various investigators.

It is of interest that healthy carriers in this study were found to be positive for a period of time considerably longer than in the one probable non-paralytic case (N.A. in family IV), whose stool was negative at the time of onset of muscle weakness. This is in keeping with an impression gained earlier in this laboratory that the well carrier maintains virus more consistently than the frank paralytic case of poliomyelitis. The question arises as to whether this individual developed poliomyelitis as a result of exposure to the healthy carrier in her family or was otherwise exposed simultaneously with her father who succumbed to the infection. Evidence for the actual development of the carrier state from another carrier is suggested in only one instance (R.T. in family II) and even here it is not ind cated whether the significant exposure was to the carrier (A.T.), to the frank case in the family, or to a common source to which all three were related. The results as a whole point out the probability of simultaneous infection of the family group, rather than serial transmission, a fact emphasized in other studies from this laboratory.

It should be remarked that although single monkeys were used for most of the specimens, the regularity with which a person was either positive or negative week after week speaks well for the efficacy of this method for detection of virus in stools. This was, moreover, a year in which virus was sharply effective in producing disease of monkeys. The several results which at first cast suspicions on the technique, *i.e.* individuals found positive then negative then positive, were verified by means of reworking the original specimens and inoculating new monkeys. Of ten such specimens which were retested, identical results were obtained with seven and the remaining three probably contained such small amounts of virus that isolation from a given sampling was chance.

If approximately one-fifth of all the familial associates of a case of poliomyelitis are carriers, (23 per cent from Table I), and if each case has from five to ten associates, the number of silent carriers in a given population must be equal to, or at most, twice, the number of cases and not 10 or a 100 times the number as theoretically advanced by some individuals. And since most of these carriers will be found among the familial or close associates (8, 10, 13, and present report) and not among the population at random (10, 16, 17)

it is increasingly evident that measures directed toward the control of infected individuals would be most profitably applied to families in which cases of the disease have been recognized.

SUMMARY AND CONCLUSIONS

The occurrence and duration of the carrier state in familial associates of recognized cases of poliomyelitis was studied by the examination for virus of stool specimens collected from the members of four families at regular intervals for a period of over 2 months. The results indicate that: (1) virus may persist in their stools continuously for 4 to 5 weeks; (2) virus may be encountered intermittently in the stools; (3) in some instances virus may be present for brief periods only; (4) children are more likely to maintain virus than are adults in the same family; (5) infection of a family takes place rapidly, suggesting again simultaneous infection from a common source.

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STUDIES ON PLANT HYPERTENSINASE*

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From the kidneys and other organs of normal animals, an enzyme, hypertensinase, has been extracted (1). This enzyme has the ability to destroy the pressor substance, hypertensin (2), probably a polypeptide, which results from the action of the enzyme on the substrate hypertensinogen, a globulin fraction in the blood plasma. Intestinal mucosa and kidney are the richest animal sources of this enzyme which is also present in practically all other animal tissues (3). An enzyme, or group of enzymes, capable of inactivating hypertensin, has also been extracted from plants, for example mushrooms (4) and yeast (5).

The present communication deals with an attempt to obtain a potent, nontoxic preparation of hypertensinase, the study of which might help to clarify the part played by this enzyme in the humoral mechanism of experimental renal hypertension.

Methods

During the search for a rich source of hypertensinase, intestinal mucosa, kidney, and plants of various kinds were extracted and compared and it was found that many plants are a much richer source than animal tissues of enzyme activity like that of hypertensinase. Aqueous extracts of jackbean meal, brewer's yeast, wheat, corn, barley, oatmeal, garlic, onion, parsley, navy and lima beans, potatoes, lentils, soya beans, and peas contained variable amounts of hypertensinase but were toxic when injected intravenously into animals. Jackbean meal, for instance, proved to be a highly potent source of hypertensinase, but it was not possible to separate the hypertensinase from the urease, either by precipitation with neutral salts, acetone, or alcohol, by changes of pH, or by inactivation of the urease by oxidation. Crystalline urease did not show any hypertensinase activity.

During the course of these experiments it was found that the bran portion of the cereal grains contained the major part of the hypertensinase activity and that extract of wheat bran yielded a potent enzyme solution which was relatively less toxic than the other plant extracts studied. Purification and con-

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centration of the wheat bran extract were therefore undertaken and the results of this study are given below.

Method of Extraction and Purification of Hypertensinase Obtained from Wheat Bran

Extraction.—25 pounds of finely ground wheat bran was thoroughly mixed with 25 pounds of washed fine sand. The mixture was moistened with distilled water and thoroughly kneaded. Care was taken to break up all lumps and still maintain the material in a moist state, so that it could be poured. It was allowed to stand for 24 hours at 3–4°C.; then the mixture was poured into a large metal percolator (6) filled with cold distilled water. The next day the mixture was allowed to percolate and the fluid was filtered through sand and cotton at the bottom of the percolator at a rate of 10 drops (about 0.05 cc.) per minute. The daily yield was kept in frozen state at −35°C. Of this dark brown extract, 1 cc. usually contained about 100 units of hypertensinase, 1 unit being the amount capable of completely inactivating one unit of hypertensin.

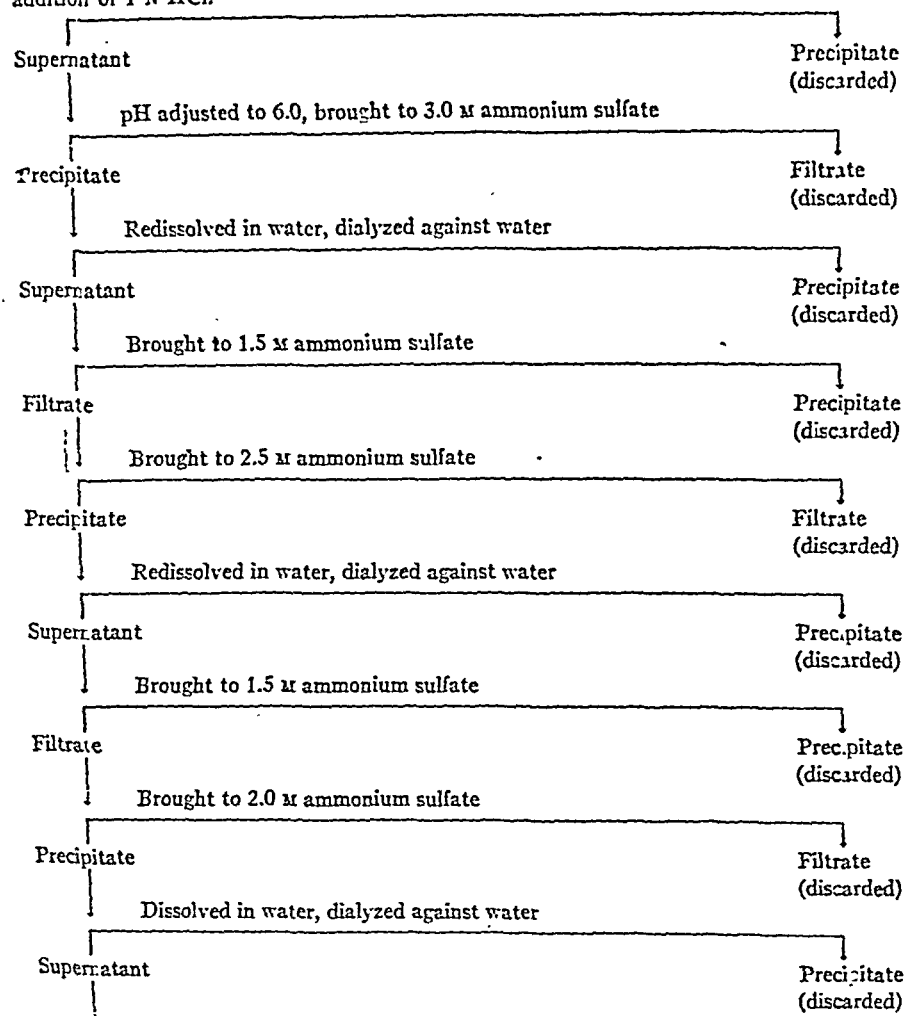
Purification.—During the entire process of purification the wheat bran proteins were maintained at a high concentration and the reagents were added slowly by capillary pipette. All procedures were carried out continuously at 1°C. and the conditions of pH and ionic strength indicated below were strictly controlled.

Five liters of the original wheat bran extract, at pH 6.2, was diluted with an equal volume of distilled water. The pH was adjusted to 4.1 by dropwise addition of 1 N HCl solution through a capillary pipette, while the mixture was vigorously stirred. The resulting suspension was allowed to settle at 0°C., overnight, after which the major portion of the clear supernatant fluid was syphoned off. The remaining portion of the mixture was centrifugalized at 0°C., the precipitate discarded, and the supernatant fluid combined with the first portion. This fluid was then adjusted to pH 6.0 by the addition of 1 N NaOH, again during vigorous stirring. To this solution was then added sufficient solid ammonium sulfate to produce a molarity of 3.0. The resulting precipitate was filtered in the cold, on large Büchner funnels, and the filtrate discarded. The precipitate was pressed, dissolved in 500 ml. of distilled water, and the resulting solution was enclosed in cellophane tubing and dialyzed in the cold against repeated changes of distilled water until free of sulfate ion. The precipitate which formed was separated by centrifugalization and discarded, and the supernatant fluid was further fractionated as follows: 4 M solution of ammonium sulfate was added gradually through a capillary pipette to bring the fluid to 1.5 M. The precipitate which formed was collected by filtration on a Büchner funnel, and discarded. The clear filtrate was then brought to 2.5 M by the addition of sufficient 4 M ammonium sulfate. The resulting precipitate was also collected, by filtration, on a Büchner funnel and the filtrate discarded. The precipitate, first pressed, then dissolved in 100 ml. of distilled water, was dialyzed against distilled water until free of sulfate. The dialysis was continued for 2 days. The euglobulin precipitate which formed was removed by centrifugalization and discarded. The clear supernatant fluid was fractionated a second time, as follows:—

The volume of 4 M ammonium sulfate required to bring the fluid to 1.5 M with respect to ammonium sulfate was calculated. The 4 M ammonium sulfate solution was then enclosed in a cellophane membrane and permitted to dialyze, with rotation, against the fluid, until equilibrium was reached. This took 48 hours. The resulting precipitate was collected on a Büchner funnel and discarded. The clear filtrate was then brought up to 2.0 M by equilibrium with the requisite amount of 4 M ammonium sulfate enclosed in a cellophane tube. Again a precipitate formed, and this was collected on a Büchner funnel, and the filtrate discarded. The precipitate was pressed, washed once with 2.0 M ammonium sulfate, dissolved

TABLE I

Flow Diagram Representing the Purification of Wheat Bran Hypertensinase



in 100 ml. of distilled water, and dialyzed free of sulfate ion against repeated changes of distilled water, in the cold. The small amount of precipitate formed in the dialysis was removed by centrifugalization and discarded. The supernatant fluid had only 24 per cent of the hypertensinase activity of the original wheat bran extract, but, on the basis of nitrogen content, was 15 times purer than the original extract.

All fractions were neutralized and rendered isotonic before they were tested for hypertensinase activity, as described below.

Table I presents a flow diagram of the steps used in the purification of the hypertensinase.

Hypertensinase Assay

Dilutions of the original preparation and of all fractions during the process of purification to be tested for hypertensinase activity were mixed with 1 cc. of physiological phosphate buffer at pH 7.8. To these mixtures 1 dog unit of hypertensin was added and the test tubes were incubated for 1 hour at 40°C. in a water bath. The reaction was stopped by placing the test tubes in an ice bath at 0°C. A dog unit of hypertensin is the amount necessary to raise the direct mean blood pressure of an unanesthetized trained dog 30 mm. Hg. The maximum rise from a dose of this size is reached in about 1 minute and the return to normal occurs in another minute or two. The samples were warmed to body temperature before injection into unanesthetized trained dogs. In these dogs, blood pressure determinations were made by the direct method, a 20 gauge needle, attached to a mercury manometer, inserted into a femoral artery. The quantity of undiluted fluid with hypertensinase-activity which completely destroyed the vasoconstrictor effect of 1 dog unit of hypertensin at 40°C. for 1 hour was considered to contain 1 unit of hypertensinase. Rabbits under the influence of nembutal, injected intravenously, also proved to be reliable test animals when 1 dog unit of hypertensin was used as the control test. In these animals the blood pressure was determined by means of a cannula, attached to a mercury manometer, inserted into a carotid artery.

Properties of Plant Hypertensinase

This enzyme is a non-dialyzable pseudoglobulin. It is destroyed at 56°C., or over, and remains active in a pH range of 3.9 to 9.5, at body temperature. Its optimum activity is at 40°C., and it is not appreciably active at 0°C. It is precipitable by neutral salts, but it is denatured by organic solvents, at room temperature, and at 0°C.

Plant hypertensinase destroys the activity of hypertensin and pepsitensin (7) if incubated with them for 1 hour at 40°C., but it does not affect the activity of renin, adrenalin, or hydroxytyramine.

Experiments with Purified Plant Hypertensinase

Intramuscular Injection.—Into a dog (No. 10-46), with experimental renal hypertension and average direct mean femoral blood pressure of 220 mm. Hg, 10 cc. of extract containing 6000 units of hypertensinase was injected intramuscularly daily for 6 days. During this period there was no reduction of the blood pressure. It was thought that the hypertensinase, injected intramuscularly, might not have been absorbed. If hypertensinase is to act on hypertensin in the plasma, it must first be absorbed into the blood stream. Therefore it was considered advisable to study the absorption of hypertensinase before further therapeutic experiments with hypertensinase were attempted.

Absorption of Hypertensinase.—Absorption studies were made on dogs and rabbits. Concentrated extract containing 2000 units of hypertensinase was injected intramuscularly into rabbits and samples of the rabbit's plasma were

titrated for hypertensinase activity by the method described above. The hypertensinase activity of the plasma did not show any increase.

On the contrary, even 2 hours after the intravenous injection of 2000 units of hypertensinase into rabbits, a fivefold increase of the hypertensinase activity of the plasma was detected. After 6 hours, the level had returned to normal. In another experiment a total of 7350 units of hypertensinase was injected intravenously into a rabbit. After 90 minutes, the rabbit's plasma still showed an almost fivefold increase of hypertensinase activity.

Similar studies were conducted on dogs. A normal dog of 8.6 kilos was given 1000 units of hypertensinase intravenously. The hypertensinase level of the plasma increased twofold, and it took 6 hours before the plasma returned to the normal level existing before the injection.

Intravenous Injection of Plant Hypertensinase into Cats and Their Reaction to Intravenous Injections of Renin and Hypertensin.—Cats were anesthetized with chloralose, 0.1 gm. per kilo body weight, and the effect on the blood pressure of a third of a dog unit of hypertensin and a third of a dog unit of renin was determined. The dog unit of hypertensin used in this study has already been defined above. The dog unit of renin is the one that has been used in all our studies. The determination of this unit was described in detail in a previous study (*6a*). It is the amount of renin necessary to raise the direct mean femoral blood pressure of at least three unanesthetized dogs 30 mm. Hg. Then the plant hypertensinase was slowly injected into the jugular vein. In a typical experiment, 750 units of hypertensinase were injected into a cat weighing 4.5 kilos. After the hypertensinase level of the plasma had increased twofold, the animal still reacted, but much less, to a unit of hypertensin, but failed to give a rise of blood pressure to 1 dog unit of renin.

Intravenous Injection of Plant Hypertensinase into Normal Dogs, and Their Reaction to Renin and Hypertensin.—The intravenous injection of the concentrated plant hypertensinase caused a sudden and great fall in blood pressure in dogs. Therefore, the material was diluted with equal parts of Ringer's solution and slowly injected by intravenous drip.

In a dog (No. 9–46) weighing 10.9 kilos, extract containing a total of 3090 units of plant hypertensinase was slowly injected during 1 hour. The comparison between the hypertensinase activity of the plasma before and after the injection showed that the hypertensinase level had increased twofold.

In a dog (No. 10–30) weighng 9.5 kilos, extract containing 3000 units of plant hypertensinase was administered intravenously in 1 hour. After the administration of 1500 units, the dog did not react to the intravenous injection of 1 unit of hypertensin and showed a blood pressure rise of only 15 mm. Hg after the intravenous injection of 3 units of renin. At the end of the injection of 3000 units of hypertensinase, 1 unit of renin, injected intravenously, produced no rise in blood pressure, 2 units of renin gave a rise of only 10 mm. Hg and 3 units of hypertensin caused a rise of only 15 mm. Hg. At this time the hypertensinase level of the plasma showed a twofold increase.

In a dog weighing 7.7 kilos (No. 10–47) a total of 2500 units of plant hypertensinase was injected intravenously in 15 minutes. After the completion of the injection, the dog reacted to 1 unit of hypertensin with a 5 mm. Hg rise of blood pressure, to 2 units with 10 mm. Hg and to 4 units with 15 mm. Hg. To 1 unit of renin the dog reacted with a 15 mm. Hg rise in blood pressure. One hour after the completion of the injection the dog reacted to the intravenous injection of 2 units of hypertensin with a rise in blood pressure of 20 mm. Hg and to 1 unit of renin with a rise of 20 mm. Hg. Four hours after the completion of the intravenous injection of the plant hypertensinase, the dog showed a normal response to the intravenous injection of 1 unit of hypertensin, with a rise in blood pressure of 30 mm. Hg, and to 1 unit of renin, with a rise of 35 mm. Hg. The plasma level of hypertensinase, which was increased twofold immediately after the injection of the plant hypertensinase, returned to the normal level 4 hours after the injection was completed.

Into a normal dog (No. 10–24) weighing 11.8 kilos an intravenous injection of plant hypertensinase was started, and, after the animal had received 2100 units of hypertensinase, during the first 25 minutes, 2 units of renin were injected intravenously, to which the dog reacted with a rise in blood pressure of 15 mm. Hg. The intravenous injection of hypertensinase was continued at a rate of 25 units per minute and, after 10 minutes, 1 unit of renin was injected intravenously, which gave a rise in blood pressure of 10 mm. Hg. After 5 minutes, 5 units of renin were injected intravenously, to which the dog responded with a 10 mm. Hg rise in blood pressure. Simultaneously with the injection of plant hypertensinase, an intravenous injection of renin was started, at a rate of half a unit of renin per minute. After 14 minutes, the dog still failed to respond to renin. An intravenous injection of 10 units of renin did not produce a rise in blood pressure. Now the rate of intravenous injection of renin was increased to 1 unit per minute. During the next 12 minutes a rise in blood pressure did not occur. Throughout the experiment a total of 4640 units of plant hypertensinase was injected intravenously. After the completion of the plant hypertensinase injection, the hypertensinase level of the plasma had increased twofold.

Into a normal dog (No. 10–41) weighing 10.4 kilos an intravenous injection of plant hypertensinase (37.5 units per cc. in Ringer's solution) was started at the rate of about 100 units of hypertensinase per minute. This rate proved to be too rapid and the injection was stopped after 5 minutes, at which time the blood pressure fell suddenly from 135 mm. Hg to 70 mm. Hg. After another 3 minutes, the blood pressure returned to its previous level and the injection was started again at a slower rate of about 30 units of hypertensinase per minute. During the next half hour enough hypertensinase was injected to add up to a total of 3500 units of hypertensinase. The blood pressure remained at its normal level of 130 to 140 mm. Hg throughout the injection, and the dog had a normal appearance. After the injection was completed, the intravenous injection of 1 unit of hypertensin gave a rise in blood pressure of 15 mm. Hg, 2 units of hypertensin a rise of 15 mm. Hg, 1 unit of renin a rise of 10 mm. Hg, 2 units of renin a rise of 5 mm. Hg, and 5 units of renin gave no rise in blood pressure. Then 200 cc. of arterial blood was withdrawn and treated for the demonstration of hypertensin (8) and hypertensinase. The hypertensinase level in the plasma had increased twofold. There was enough hypertensin present in 200 cc. of plasma to give a rise in blood pressure of 55 mm. Hg when this was injected intravenously into a normal unanesthetized trained dog.

Intravenous Infusion of Plant Hypertensinase into a Hypertensive Dog.—

A dog (No. 10–29) weighing 10 kilos, with experimental renal hypertension and direct mean femoral blood pressure of 180 mm. Hg, received an intravenous injection of plant hypertensinase. The solution contained 50 units of hypertensinase per cc. and, at the slow rate of 1 cc. per minute, a total of 3000 units of hypertensinase was injected. After the injection of

2300 units of hypertensinase, the blood pressure had changed from 180 mm. Hg to 165 mm. Hg. After the injection of 2675 units of hypertensinase the blood pressure had reached the normal level of 120 mm. Hg. At the end of the injection the blood pressure was 130 mm. Hg. After the injection was stopped, the dog reacted to the intravenous injection of 1 unit of hypertensin with a rise in blood pressure of 5 mm. Hg, to the injection of 5 units of hypertensin with a rise of 15 mm. Hg, to the injection of 1 unit of renin with no rise, and to the injection of 5 units of renin with a rise of 25 mm. Hg. At the height of the rise, 200 cc. of arterial blood was withdrawn and treated for the demonstration of hypertensin and hypertensinase in the plasma. After the injection of the 6 units of renin into the hypertensive dog treated with 3000 units of plant hypertensinase by intravenous injection, 200 cc. of blood contained enough hypertensin to give a rise in blood pressure of 50 mm. Hg (intravenous injection into a normal unanesthetized trained dog). The hypertensinase level of the blood was increased threefold. Four hours after the injection of plant hypertensinase had been stopped, the hypertensinase had returned to its normal level and the blood pressure was up to its previous hypertensive level.

Intravenous Injection of Inactivated Plant Hypertensinase into a Hypertensive Dog.—

The plant hypertensinase preparation was inactivated by incubation at 37°C. for 1 hour at pH 11. The pH was then lowered to 8.1 by the slow addition of 1/10 N HCl. Instead of 30 units of hypertensinase per cc. the solution now contained barely 1 unit per cc.

A hypertensive dog (No. 8–82) weighing 10 kilos, with a blood pressure of 200 mm. Hg received by slow intravenous injection, a quantity of inactivated extract representing 3000 units of plant hypertensinase. Throughout the injection, which lasted 75 minutes, the blood pressure of the dog did not change. After the injection, the dog reacted in a normal way to the intravenous injection of 0.05 cc. of 1:1000 adrenalin, with a rise in blood pressure of 60 mm. Hg, to the injection of 1 unit of hypertensin with a rise of 20 mm. Hg, and to the injection of 1 unit of renin with a rise of 25 mm. Hg. Then 5 units of renin were injected intravenously and, at the height of the rise of blood pressure, 200 cc. of arterial blood was withdrawn, to be treated for the demonstration of hypertensin and hypertensinase. The plasma of this sample contained enough hypertensin to give a rise of 30 mm. Hg in a normal unanesthetized dog. The hypertensinase level of the blood was not increased after the intravenous injection of 3000 units of inactivated plant hypertensinase.

DISCUSSION

There is no proof that the enzyme hypertensinase is involved in the humoral mechanism of renal hypertension. Blood plasma does contain hypertensinase, and the kidney contains a large amount of it, rating only second to intestinal mucosa (3). An indication that the kidney is the main source of hypertensinase in the blood plasma is the almost complete disappearance of hypertensinase from the latter, after bilateral nephrectomy (3); but this finding has been questioned (9). It has been asserted that renal venous blood, from an ischemic kidney, contains less hypertensinase than blood from the renal vein of a normal kidney (10), but this we have not been able to confirm, and it has not been confirmed by other investigators (11). In addition, the importance of hypertensinase is rendered questionable by the fact that the hypertensinase content of the plasma of hypertensive dogs has always been found normal (9).

Because hypertensinase is the only known substance in the blood which has the ability to destroy hypertensin *in vitro*, almost all attempts to treat renal hypertension by injection of organ extracts have been based on the idea of supplying the enzyme which destroys the pressor substance, hypertensin, although a lack of hypertensinase has never been established in renal hypertension. Studies of the action *in vivo* of hypertensinase made from the kidney have not been possible because of the relatively low potency and impurity of the preparations which usually contained more renin than hypertensinase.

With the preparation of a purified and highly potent plant hypertensinase, which does not contain renin, the attempt has been made to gain more knowledge about the antipressor properties of this enzyme. By our simple method for the determination of the potency of the hypertensinase, it has been possible to follow the possible absorption of hypertensinase after injection at various sites in the body.

The properties of the wheat bran hypertensinase used in our experiments were found to be similar to those of the enzyme solution obtained from yeast cells (12). This enzyme destroys hypertensin and pepsitensin, and the authors described it as an aminopolypeptidase. We did not make an exhaustive study of the chemical properties of the enzyme solution used in our experiments. The fact that it did not destroy adrenalin or hydroxytyramin excluded the action of a phenolic oxidase. It is probable that the action is not that of an oxidase but of a proteolytic enzyme acting by hydrolysis.

Failure to raise the blood level of hypertensinase and to reduce blood pressure of hypertensive dogs by the intramuscular injection of large amounts of plant hypertensinase is of interest because the intramuscular injection of renal extract has been used as a treatment for hypertension. Page even used the hypertensinase titer as an indicator of the activity of his renal extracts, which were administered by intramuscular injection. The renal extract of Grollman and his collaborators (13) could not contain hypertensinase, because this was undoubtedly destroyed during the preparation.

Helmer, Kohlstaedt, Kempf, and Page (14) found that most of the material in their antipressor extract which was capable of destroying hypertensin was in the same range of ammonium sulfate precipitability as the material responsible for the antipressor activity, when assayed on hypertensive dogs and rats. The reduction of arterial blood pressure of hypertensive patients and animals with extracts of kidney (15) was explained therefore as due chiefly to their ability to destroy hypertensin (16). It is difficult to evaluate the results of their treatment with kidney extracts because the authors state also that the preparation of active extract is almost a hit or miss problem and that expressions such as "specific" and "non-specific" would only confuse the issue. Goldblatt, Kahn, and Lewis (17), with a kidney extract made in accordance with the details given by Page and collaborators, failed to reduce the blood pressure of hypertensive dogs. The kidney extract prepared by Schales,

Stead, and Warren (18) did contain a goodly amount of hypertensinase, because 1 liter inactivated the amount of hypertensin produced by incubating 1 to 3 liters of beef serum with an excess of renin under optimum conditions. In five of their seven patients, there was a significant lowering of the high blood pressure, which appeared to be related to the severe local and general reactions. Because the intramuscular injection of an extract poor in hypertensinase also produced a fall in blood pressure, similar to that produced by the unmodified extract, the authors concluded that the blood pressure-lowering effect of their renal extract was due to a general toxic effect of the crude tissue extract rather than to specific interference with the renin-hypertensin mechanism. In these experiments, too, the effect cannot be attributed to hypertensinase which, as we have shown above, is not absorbed into the blood stream from an intramuscular injection.

The intravenous injection of large amounts of plant hypertensinase gave us the opportunity to study the direct action of hypertensinase on renin and hypertensin *in vivo*. In the circulating blood, hypertensinase interferes not only with the action of hypertensin, as in the test tube, but it also inhibits the effect of renin. The impression is gained that the action on renin is even greater than on hypertensin. This may be explained by the slow enzymatic action of the renin and hypertensinase, two antagonistic enzymes, which allows better opportunity for the destruction of the end product, hypertensin, formed in the blood. Hypertensin, however, injected intravenously, acts so quickly that it is only slightly affected by the slowly acting hypertensinase of the blood. To produce effects, the level of hypertensinase in the plasma must be greatly increased, and the best results were obtained when it was increased at least threefold above normal. When no increase was produced (as by the injection of inactivated extracts) both renin and hypertensin retained their normal activity.

In brief experiments, the intravenous injection of plant hypertensinase lowered the blood pressure of dogs with experimental renal hypertension to normal levels. Inactivated plant hypertensinase failed to produce this effect. The lowering of the blood pressure was temporary and lasted only as long as the hypertensinase level was increased. Within 4 hours after the injection of 3000 units of plant hypertensinase into a dog of 10 kilos, the blood pressure returned to its original level.

The demonstration that the pressor substance hypertensin is still formed in the blood after the injection of plant hypertensinase can be explained on the assumption that this enzyme does not quickly destroy all the pressor substance formed.

SUMMARY

Many plants contain an enzyme similar in most biological properties to the hypertensinase obtained from blood and some animal tissues, notably kidney and intestinal mucosa.

Wheat bran is a rich source of the plant hypertensinase, and from it a potent, non-toxic preparation was made by the use of isoelectric and ammonium sulfate precipitation as the means of purification.

Hypertensinase derived from bran and administered intramuscularly was not absorbed, or was absorbed only very slowly, into the blood plasma.

Repeated intramuscular injection of large quantities of plant hypertensinase did not reduce the blood pressure of dogs with experimental renal hypertension.

The intravenous injection of large quantities of plant hypertensinase into dogs resulted in an immediate increase in the content of hypertensinase in the plasma.

Dogs with a high hypertensinase level in the plasma failed to react, or reacted much less markedly to the intravenous injection of amounts of renin or hypertensin which had previously proved effective.

The slow intravenous injection of plant hypertensinase into a dog with experimental renal hypertension reduced the blood pressure to the normal level for the period during which the concentration of plant hypertensinase in the blood was considerably elevated. After the return of the hypertensinase of the plasma to normal, the blood pressure rose again to its previously high level.

Inactivated plant hypertensinase did not increase the hypertensinase content of the plasma, did not interfere with the action of renin and hypertensin, and did not reduce the high blood pressure of dogs with experimental renal hypertension.

In a dog with an increased level of plasma hypertensinase, the pressor substance hypertensin could still be detected in the systemic blood immediately after the intravenous injection of renin in an amount to which the animal responded with only a slight rise in blood pressure.

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STUDIES IN THE RELATION OF THE HEMOLYTIC STREPTOCOCCUS TO RHEUMATIC FEVER

II. Fractionation of the Hemolytic Streptococcus by High Speed Centrifugation*

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The literature on serologic investigations of rheumatic fever by streptococcal substances has been reviewed elsewhere (1). It was noted there that the sera of patients with acute rheumatic fever react similarly to those of patients convalescing from streptococcal infections with respect to two streptococcal antigens, the hemolysin and the fibrinolysin. Although these data have added serologic support to the epidemiologic evidence for a relation between the hemolytic streptococcus and rheumatic fever, they have failed to identify either of these streptococcal products as possible agents in the pathogenesis of rheumatic fever. It was pointed out, however, that the failure of such indentification in the case of two streptococcal antigens, out of the considerable number of such antigens which exist, leaves a wide area of possible immunologic relation. Accordingly, the present studies involve a systematic exploration of the immunologically distinct constituents and products of the hemolytic streptococcus, in an attempt either to identify or to rule out a streptococcal agent or mechanism as playing a part in rheumatic fever.

The first step in the immunologic phase of this work has been a reexamination of the antigenic structure of the hemolytic streptococcus. Of its constituent antigens, those most clearly defined and best studied have been the ones concerned in serologic classification: the type-specific M protein by Lancefield (2, 3) and by Zittle (4), the group-specific carbohydrate by Lancefield (5) and by Zittle and Harris (6), and the type-specific T substance by Lancefield and her collaborators (7). The remaining constituents, which comprise the bulk of this organism, have, however, been less clearly defined. There have been three major attacks on this problem, that of Lancefield, of Heidelberger *et al.*, and of Mudd and coworkers. Lancefield (3, 8) found a broadly specific nucleoprotein, "P," in the substance of the streptococcal cell and suspected, but was unable to prove, the presence of another protein (3, 9). Heidelberger *et al.* (10–13) obtained chemical fractions lettered from D to K. Of these, the E to K fractions

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all showed serologic cross-reaction, and absorbed antibodies to all the fractions from antistreptococcal sera. The D fraction did not, however, absorb out antibodies to the E and K fractions. All of these contained nucleic acid. Mudd and Lackman (14) found a single nucleoprotein in the sonic extract of the hemolytic streptococcus, which was shown by Mudd and Wiener (15) to have broad specificity. It was shown by Zittle (4) that this fraction gave tests for both ribose and desoxyribose nucleic acids. Sevag *et al.* (16) isolated heavy particles from sonic extracts of hemolytic streptococcus by high speed centrifugation. They found that the particles were antigenic in rabbits and appeared to contain the group-specific carbohydrate.

The first step in our investigations was to reexamine the constitution of the hemolytic streptococcus so that each distinct antigen comprising the organism could be studied with respect to the immunologic reactions of the rheumatic subjects. The means adopted was that of physical separation of the constituents of the organism rather than chemical fractionation.

Methods and Materials

The streptococci used in these studies included the following strains of group A: NY 5, a strain widely used for the production of erythrogenic toxin; C 203, a type 1 strain also isolated from a patient with scarlet fever; 1048 M, a strain which maintains its production of the type-specific antigen in the laboratory; and H 44, a hyaluronidase-producing strain.[1] The organisms were cultivated in dextrose broth or tryptose phosphate broth with added sodium bicarbonate and phenol red. Some cultures were grown in protein-free medium which was essentially a dialysate of Difco dextrose broth. In addition, large amounts of streptococcal cell sediment (strain NY 5) were made available through the courtesy of the Lederle Laboratories and of Parke, Davis and Company.

The cultures of streptococci were incubated overnight at 37°C. and then neutralized with 4 N sodium hydroxide and reincubated. This was continued until there was no evidence of increased growth. The organisms were then collected by centrifugation, washed twice in physiologic saline solution, and suspended in distilled water at a final concentration usually corresponding to 0.03 gm. of dry weight per cc. This suspension was subjected to high frequency sonic vibration in a magneto-striction oscillator[2] (17) in order to disintegrate the organisms. The milky suspension which resulted was further diluted with one volume of distilled water, aliquots were removed for determination of the total concentration of the suspension in terms of dry weight per cc., and the material was subjected to differential centrifugation. The high speed centrifugation was done in a motor driven centrifuge run in an evacuated chamber (18).

Complement fixation tests were done by incubating the dilution of serum in a volume of 0.4 cc. with 0.1 cc. of the dilution of antigen and 0.1 cc. of complement. The dilution of complement was such that 0.1 cc. contained 1.3 to 1.5 units, under the conditions of the test. After incubation of the mixture at 37°C. for 45 minutes, 0.1 cc. of rabbit anti-sheep–erythrocyte serum, diluted 1:1000, and 0.1 cc. of a 4 per cent suspension of sheep erythrocytes were

[1] The first three of these strains were from the collection of the Department of Bacteriology, School of Medicine, University of Pennsylvania. For the subculture of the H 44 strain the author is indebted to Dr. Karl Meyer.

[2] Manufactured by the Raytheon Manufacturing Company, Boston.

added to each tube. The tubes were incubated at 37°C. for 30 minutes, and the degrees of hemolysis were read as 0, trace, weak, strong, and complete.

Analyses for total nitrogen were done by the micro-Kjeldahl technic, using a $CuSO\text{-}K_2SO_4\text{-}H_2SO_4$ digestion mixture, with superoxol to complete the digestion. The indicator was a mixture of methyl red and methylene blue. Seventieth normal hydrochloric acid was used for the titration, and a steel-gray color was taken as the end-point. The analyses for total phosphorus were done by the method of King (19). It was found necessary, however, to add the molybdate reagent and the sulfonic acid in succession and then to read the color immediately in the photoelectric colorimeter in order to avoid non-specific increments of color. Quantitative estimations of desoxyribose nucleic acid were performed by the method of Dische (20) and measurement of ribonucleic acid by that of Mejbaum (21).

RESULTS

The Fractions.—As a result of experimentation with speeds and lengths of time of centrifugation of suspensions of disintegrated streptococci it was found that an insoluble residue (R) was brought down by centrifugation at 5000 R.P.M. for 30 minutes. The very few organisms which had not been disintegrated were completely removed in this step. The supernate of this centrifugation was a highly opalescent fluid. If this was subjected to repeated centrifugation for periods of 1 hour, first at 15,000 R.P.M. and then at 30,000 R.P.M., a series of opalescent yellowish pellets was obtained, which could be resuspended into a white opalescent suspension. The pellets decreased markedly in size after the 3rd hour at 30,000 R.P.M. (90,000 G) but minute pellets continued to appear even after 9 successive 1 hour centrifugations. Particles of the same range of size as those comprising these pellets had been identified in the cytoplasm of cells of various organs, primarily by Claude (22). In conformity with his nomenclature they were named cytoplasmic particles (CP). The supernate from 8 hours of centrifugation at 30,000 R.P.M. was found to contain a considerable amount of protein. This fraction, after dialysis to remove small molecules, was designated the supernate (S). Since there was both electrophoretic and immunologic evidence that the later pellets obtained at 30,000 R.P.M. were mixtures of the CP and S, only the first two sets of pellets derived at that speed were included with the pellets of the centrifugation at 15,000 R.P.M. in the preparation of the cytoplasmic particles.

It was found that complete separation of the CP and S by centrifugation was impossible. In the case of the CP, any repeated centrifugation in an attempt to purify the material resulted in loss of stability in suspension. On the other hand the smallest units with the immunologic and chemical characteristics of CP could be brought down only by centrifugal fields which sedimented some of the S fraction. In the work to be reported here each of the fractions was not entirely free of the other.

Serologic Reactions of the Fractions

Both soluble fractions reacted with antistreptococcal sera in precipitation and complement fixation tests. The differences between the serologic character of

the CP and S fractions are better shown by their characteristics in complement fixation tests, of which a typical example is shown in Table I.

Complement Fixation.—Since nothing was known of the serologic reactions of these fractions, each was tested against antistreptococcal sera by complement fixation tests in which both antiserum and antigen were progressively diluted.

TABLE I

Complement Fixation of the Streptococcal Nucleoproteins with Rheumatic and Antistreptococcal Sera

	Serum R 26 (rabbit antistreptococcal)									Serum 1268 (active rheumatic)									
	Dilution 1:									Dilution 1:									
	8	16	32	64	128	256	512	1024	2048	4	8	16	32	64	128	256	512	1024	C
CP																			
0.02% sol.			0	0	0	0	0	w	s			0	0	0	0	0	w	s	ac
0.01			0	0	0	0	0	tr	s			0	0	0	0	0	tr	s	c
0.005			0	0	0	0	0	tr	w			0	0	0	0	0	tr	s	c
0.0025			0	0	0	0	0	tr	s			0	0	0	0	0	0	s	c
0.0013			w	w	w	w	w	s	s			w	w	w	w	s	s	s	c
0.0006			s	s	s	s	s	s	s			s	s	s	ac	s	ac	ac	c
S																			
0.2% sol.	0	0	s	s	c	c				0	0	w	s	ac	c				c
0.1	0	0	tr	s	ac	c				0	0	0	s	c	c				c
0.05	0	0	tr	s	c	c				0	0	tr	s	c	c				c
0.025	0	0	w	s	c	c				0	tr	s	s	c	c				c
0.013	0	tr	w	s	c	c				w	s	c	c	c	c				c
0.065	tr	s	c	c	c	c				ac	c	c	c	c	c				c
C	ac	c								c	c								

0 = no hemolysis.
tr = trace of hemolysis.
w = weak hemolysis.
s = strong hemolysis.
ac = hemolysis almost complete.
c = complete hemolysis.

Such a two-way test, as shown in Table I, demonstrates the serologic behavior of the two fractions. It is seen in this table that the optimal dilution of antigen—the dilution at which the antiserum shows its full titer—is twenty times as high for CP as for S. The titer of the serum against CP is considerably higher than that against S, and the area of complement fixation differs in shape between the two antigens.

The optimum antigen concentrations of each of the two fractions in the complement fixation test were found to be constant when they were tested against

weak and strong sera, so that these concentrations represent an immunologic characteristic of the respective fractions.

Antibodies to both these fractions were found in all sera of rabbits immunized with whole streptococcal cells. They were found also in the sera of a majority of patients convalescing from acute hemolytic streptococcal infections and of patients with acute or subsiding rheumatic infection. Such antibodies were found also in apparently normal subjects, although generally in a lower range of titer than in the poststreptococcal or rheumatic patients. These data are included in the succeeding paper.

TABLE II

Complement Fixation of CP and S Fractions with Antisera to Other Strains of Group A Hemolytic Streptococcus and to Related Organisms

(*Antigens Derived from NY 5 Strain*)

Strain	Classification	Anti-CP titer	Anti-S titer	Anticomplementary control Serum 1:4
NY 5	Group A	256	64	c
1048 M	" "	64	32	c
1048 M	" "	1024	128	ac
K 151	Group B	<4	<4	c
K 151	" "	8	<4	c
K 151	" "	32	32	c
K 151	" "	32	32	ac
O 90	" "	4	<4	ac
O 90	" "	6	4	c
	Streptococcus viridans	6	6	c
	Smooth pneumococcus	16	32	c
		4	<4	c
		8	16	ac

Breadth of Specificity.—Both the CP and the S fractions gave evidence of broad specificity within group A by reacting with almost all normal human sera, and with almost all sera of patients with streptococcal or rheumatic infections, at their respective ranges of titer. There were also cross-reactions among rabbit antistreptococcal sera of different type-specificity and among anti-CP and anti-S sera of rabbits immunized with fractions derived from streptococci of other types.

In order to detect wider ranges of cross-reaction, complement fixation tests were performed between CP and S prepared from a group A strain and sera of rabbits immunized with group B streptococci, *Streptococcus viridans*, and smooth pneumococci. The results of some of these titrations are shown in Table II. The results show that of each set of non-group A sera at least one

serum reacts with the CP or S derived from group A streptococci, although in a lower range of titer than in the case of group A sera. Both fractions, then, have wide reactivity.

Serologic Distinction of the CP and S Fractions.—The difference between the cytoplasmic particles and the S fraction was indicated by the difference in optimal antigen titer, the shape of the complement fixation area in the optimal proportions test, and the difference in titers of given sera *versus* CP and S. The latter showed considerable variation. The titers of most sera tested were higher against the CP than against S, and the observed difference ranged from a ratio of 16 to 1. As an additional test of the serologic distinction of S from CP, a suspension of the latter was subjected to sonic oscillation and subsequent centrifugation. The supernate of this preparation did not show the serologic characteristics of S, indicating that S was not a product of disintegration of CP.

The final proof of serologic difference is, however, given by the cross-absorption test. In the classical form of the cross-absorption test the immunologic distinction between two antigens is demonstrated as follows: A serum containing antibodies to both antigens is treated with successive aliquots of one antigen until a further addition produces no precipitate. If an addition of the other antigen to this absorbed specimen of serum now produces a specific precipitate, and if the entire experiment can be repeated for another aliquot of the serum with the rôles of the two antigens reversed, the difference between the antigens has been demonstrated. This form of the cross-absorption test was not feasible in the case of CP and S, since repeated additions of each absorbing fraction continued to precipitate small amounts of antibody from antistreptococcal serum. This was probably due to slight contamination of each fraction with the other. Consequently complement fixation tests were used to measure antibodies to each fraction in sera absorbed with various quantities of either preparation. Absorption tests were set up as follows:—

Constant amounts of a given rabbit antistreptococcal serum or of serum from a rheumatic subject were incubated with varying amounts of CP. A similar row of tubes was set up with appropriate concentrations of S. After incubation at 37°C. for 1 hour, and overnight in the refrigerator, each tube was subjected to centrifugation for an hour at 32,000 R.P.M. and the supernate was used in complement fixation tests. A typical absorption experiment is shown in Table III.

This table shows that each fraction, at several concentrations, absorbed from the serum more antibody to itself than to the other fraction.

Immunization Experiments.—The immunization of rabbits with both CP and S fractions was undertaken. In order to simplify the procedure technically, and in view of the difficulty frequently encountered in immunization with isolated bacterial proteins, the technic of Freund and Bonanto (23) was employed.

Each rabbit immunized with CP received 20 mg. of this material dissolved in 1.0 cc. of saline solution. This solution of protein had been emulsified in 1.0 cc. of falba and 4.0 cc. of

mineral oil. The resulting 6.0 cc. of emulsion was injected into two sites on the back of the rabbit. The emulsion of S was treated similarly.

The results of the immunization with CP were quite good. Measurable antibodies appeared within 2 weeks and rose to titers almost as high as those obtained on immunizing rabbits with repeated injections of whole streptococci. These sera were free of anti-S antibodies. In the case of the rabbits immunized with S, antibodies were found to S, although in lower titer than the anti-CP

TABLE III

Cross-Absorption Test of the CP and S Fractions. Human Serum (Rheumatic Fever) Complement Fixation Test

Absorbed with	Tested with												
	CP 1:						S 1:						0
	8	16	32	64	128	256	8	16	32	64	128	256	
CP													
1.6 cc.	c	c	c	c	c	c	s	c	c	c	c	c	c
0.8 "	s	c	c	c	c	c	0	w	c	c	c	c	c
0.4 "	0	s	c	c	c	c	0	w	c	c	c	c	c
0.2 "	0	0	0	s	c	c	0	0	c	c	c	c	c
S													
1.6 cc.	0	w	s	c	c	c	c	c	c	c	c	c	c
0.8 "	0	0	c	c	c	c	s	c	c	c	c	c	c
0.4 "	0	0	w	c	c	c	0	w	c	c	c	c	c
0.2 "	0	0	0	w	c	c	0	w	s	c	c	c	c
O	0	0	0	0	w	c	0	0	s	c	c	c	c

S used in 0.4 per cent solution for absorption.
CP used in 0.8 per cent solution for absorption.

titer of the anti-CP sera. Moreover, it was found that the anti-S sera showed antibodies to CP as well. This was attributed to the known contamination of S by the CP, and to the fact that the latter is more potent antigenically.

Chemical Properties of the Fractions

Analyses for Nitrogen and Phosphorous.—Specimens of CP and S as prepared from several strains of group A streptococci were analyzed for total nitrogen and phosphorus content. Typical results of such analyses, shown in Table IV, show that the nitrogen content of both fractions is consistent with that of protein, and that the percentage of nitrogen in S is higher than that in CP, although some variation is noted from preparation to preparation.

Nucleoprotein Characterization.—Because phosphorus was found in both CP and S preparations, nucleoprotein tests were performed.

The beta-naphthol test was done according to Steudel (24): One cc. of a 0.3 per cent solution of beta-naphthol in concentrated sulfuric acid was overlaid with an equal amount of a 1 per cent solution of the material to be tested, and the tube was agitated slightly. In the case of the CP a deep, reddish brown ring appeared at the liquid interface, whereas preparations of S produced a blue ring.

These two color reactions are characteristic of ribose nucleic acid and of desoxyribose nucleic acid respectively. On further testing, the predominance of ribose nucleic acid in the CP was confirmed by the orcinol reaction and that of desoxyribose nucleic acid in S by the diphenylamine test. Quantitative

TABLE IV

Typical Results of Analysis of CP and S Fractions of Group A Hemolytic Streptococci for Total Nitrogen, Total Phosphorus, and Type of Nucleic Acid

Strain	Preparation	Time of vibration	CP			S		
			N	P	N. A.	N	P	N.A.
		hrs.	*per cent*	*per cent*		*per cent*	*per cent*	
NY 5	5	1	9.5	0.45	RNA*	12.0	1.78	DNA‡
NY 5	8	1	10.8	0.54	"	11.0	1.52	"
NY 5	13	1	11.8	0.70	"	13.0	2.05	"
NY 5	13	4	11.4	0.64	"	12.6	1.85	"
NY 5	14	1	10.3	0.76	"	12.3	1.1	"
NY 5	14	3	9.4	0.59	"	13.0	1.8	"
H 44	4	1	11.5	0.80	"	12.3	2.7	"
H 44	4	4	10.3	0.88	"	11.0	2.5	"
C 203 M	1	1	8.9	0.70	"	10.0	2.1	"
1048 M	3	1	10.9	0.68	"	12.2	2.4	"

* Predominantly ribose nucleic acid.

‡ Predominantly desoxyribose nucleic acid.

analyses of all the CP and S specimens were made by the Mejbaum test for ribose nucleic acid and the Dische reaction for desoxyribose nucleic acid. These will be reported elsewhere (25).

Electrophoresis and Ultracentrifugation.—A number of specimens of both soluble fractions were tested by electrophoresis, and one specimen of each fraction by ultracentrifugation. The CP fraction showed a single boundary by electrophoresis, with a mobility of -4.4×10^{-5} at a pH of 7.4. Since the material was quite opaque the electrophoretic uniformity was determined by examination of both ascending and descending boundaries. A minute contaminant of an electrophoretic mobility corresponding to the chief component of S was noted. In the ultracentrifuge the cytoplasmic particles were shown to be of a very wide range of size. At 19,000 R.P.M. the chamber was cleared in a few minutes, after showing a continuous band of particle sizes across the entire field.

Examination of the S fraction by electrophoresis revealed two major components, presumably protein, one in approximately twice the concentration

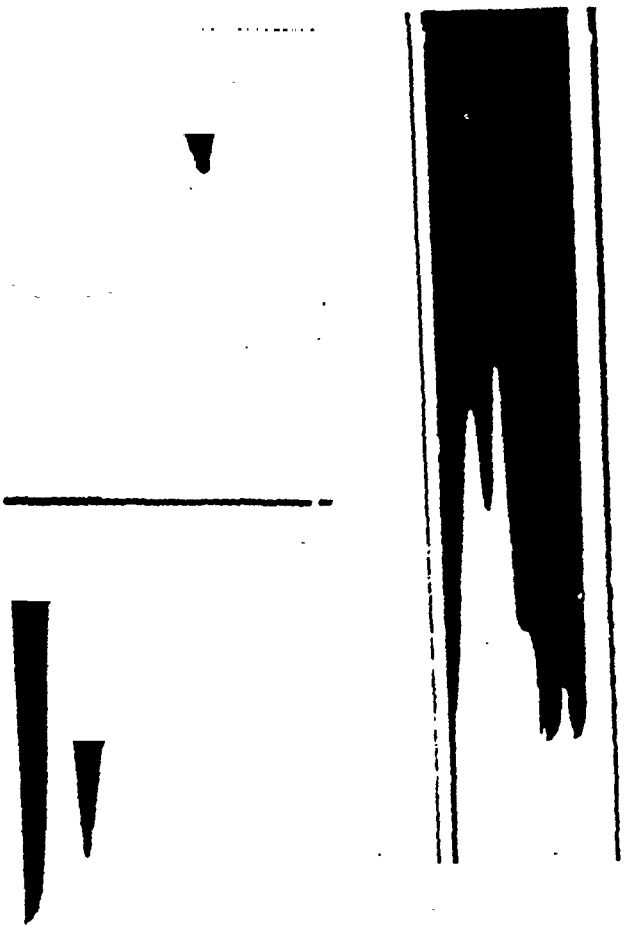

FIG. 1. Electrophoretic (descending and ascending) and ultracentrifugal patterns of a typical preparation of S (strain NY 5). Electrophoresis of a 3.6 per cent solution in phosphate buffer, pH 7.4, $\mu = 0.02$, 25 ma. Ultracentrifugation at same concentration and pH, at a field strength of 170,000 (48,000 R.P.M.).

of the other. These were flanked by a trace of a slower component, which had the mobility of the CP fraction, and a more rapid component, whose mobility was consistent with that of nucleic acid. A typical pattern is shown in Fig. 1. The mobilities of the major components, when the S fraction was tested at a concentration of 3.6 per cent and pH 7.4, are shown in Table V. At a con-

centration of 1 per cent the mobilities were some what higher, at that pH, 7.1 and 5.8, respectively. The same two components, at the same mobilities and relative concentrations, were found on examination of specimens of S prepared from streptococci of strain H 44 and strain 1048.

Ultracentrifugation of S at the same concentration and pH, at a field strength of 170,000 (48,000 R.P.M.) also showed two major components, one of which was approximately at twice the concentration of the other. It was considered probable that the two components were the same by both means of analysis. On this assumption the characteristics of the two components of S are summarized in Table V. The corresponding patterns are shown in Fig. 1. Table V shows quite similar relative concentrations for the larger and smaller protein components of S as determined by the two physical means of examination. On repetition of the ultracentrifugation at approximately one-tenth the con-

TABLE V

Electrophoretic and Ultracentrifugal Data on the Two Protein Components of the S Fraction

	Electrophoresis		Ultracentrifugation	
	Mobility	Percentage	Sedimentation constant	Percentage
Larger component	−6.7	62.9	4.5	63.3
Smaller component	−5.2	37.1	6.9	36.7

centration used in the earlier run, the two components showed the same sedimentation constants, although the apparent relative concentrations were 86.2 per cent and 13.8 per cent, respectively.

The Structure of the Cytoplasmic Particles.—Analyses of lyophilized preparations of CP were made for lipoids and carbohydrates.

150 mg. of the dried material was extracted three times in a mixture of 25 cc. of ethyl ether and 25 cc. of 95 per cent ethyl alcohol. Each extraction consisted of one-half hour of refluxing on an electric hot plate. The three successive extracts were added and concentrated *in vacuo* to a small volume, which was then divided for determinations of dry weight, nitrogen, and phosphorus.

It was found that the total lipoid extracted by this method ranged from 10.1 to 13.4 per cent. The average nitrogen content of the lipoid extract was 3.4 per cent, and that of phosphorus, 0.5 per cent.

Other aliquots of lyophilized CP were analyzed for the group-specific carbohydrate by the method of Zittle and Harris (6). It was found that 1 per cent of the lyophilized CP was crude carbohydrate. This was consistent with a similar range of yield of carbohydrate from the whole organism, since it will be shown below that the CP is by far the major constituent of the hemolytic streptococcus.

Attempts were made to separate the nucleic acid portion of the nucleoprotein in the CP from its protein by salting out. In contrast to similar experiments performed with S, these attempts were unsuccessful, indicating that the nucleoprotein in CP was a probably true compound with non-polar bonds between protein and nucleic acid.

The Structure of the S Fraction.—The electrophoretic pattern had indicated that the S fraction contained two proteins. Either one or both might be the protein part of the nucleoprotein identified earlier by chemical means. The separation of a component of a mobility consistent with that of nucleic acid in the electrophoretic cell indicated that the nucleoprotein might be of the

TABLE VI

Nitrogen, Phosphorus, and Desoxyribose Nucleic Acid Content of the Dissociation Products of S

Strain	Preparation	Original S		Sediment of ammonium sulfate precipitation		Supernate of ammonium sulfate precipitation	
		N	P	N	P	N	P
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
NY 5	3	11.1	2.47	9.6	0.70	9.8	2.38
NY 5	4	13.0	2.05	12.1	0.75	11.8	2.20
NY 5	5	12.6	1.78	12.4	0.13*	12.0	1.75
NY 5	8	12.4	2.80	11.6	0.09	11.8	2.52
NY 5	13	13.2	2.55	12.2	0.64	9.6	2.40
H 44	1	12.1	2.93	12.8	0.80	12.1	2.75
C 203 M	1	10.6	2.86	10.7	1.08	10.0	2.33

* Supernate of two successive precipitations with ammonium sulfate.

dissociable, or protein-nucleate type (26). This hint was confirmed by salting out experiments.

To a solution of S was added three volumes of a cold saturated neutral solution of ammonium sulfate. The redissolved precipitate and its supernate were dialyzed free of ammonium ions, lyophilized, and analyzed for nitrogen and phosphorus.

The results of such experiments, some of which are summarized in Table VI, show that phosphorus was largely lost in the course of the manipulation and that more of it appeared in the supernate, suggesting a dissociation of protein and nucleic acid. More specific evidence of such dissociation was offered by the fact that the desoxyribose nucleic acid content of the supernate was in all cases at least five times as high as that of the sediment. The DNA content thus determined did not account for all the phosphorus found in supernate, since the phosphorus concentration of nucleic acid is 10 per cent, so that phosphorus-containing material other than nucleic acid was present in this fraction. Since the N:P ratio of the nucleic acids is approximately 1.5:1, the relative nitrogen and phosphorus content of the supernate shows that portions other

than nucleic acid were separated from S by the treatment with ammonium sulfate. At least one of the proteins in S is, then, a protein nucleate. It is not possible to say at present whether the other component is of similar nature or is a simple protein.

No attempts to isolate the two protein components of S have been made as yet.

TABLE VII

Typical Relative Yields of R, CP, and S on Varying the Time of Sonic Vibration of Hemolytic Streptococci

Strain	Preparation	Time of vibration	Total dry weight of organisms	R recovered	CP recovered	S recovered
		hrs.	*mg.*	*mg.*	*mg.*	*mg.*
NY 5	13	1	840	420	350	85
NY 5	13	2	820	290	385	87
NY 5	13	3	810	220	650	85
NY 5	13	4	810	90	790	97
NY 5	14	1	500	207	95	54
NY 5	14	1	2100	920	880	127
NY 5	14	2	2200	480	1320	152
NY 5	14	3	2200	160	1560	148
NY 5	14	4	2100	160	1600	164
H 44	4	1	288	72.5	75	84
H 44	4	1	1220	250	292	282
H 44	4	4	1220	85	390	289

The difference between the sum of the fractions and the total weight of organisms is accounted for largely by the four sets of pellets which were known to be mixtures of CP and S, after collecting the pellets included in CP, and to a smaller extent by the dialyzable constituents of the streptococcus.

The Structure of the Hemolytic Streptococcus

The Relation of the Known Antigens to These Fractions.—The presence of the group-specific carbohydrate, or C substance, in the cytoplasmic particle has been noted above. Similar attempts at extraction of the carbohydrate from R (the insoluble residue left by disintegration) and from S failed. It was concluded that the carbohydrate was entirely contained in the cytoplasmic particles.

The type-specific M antigen was obtained from the R fraction by both the Lancefield and Zittle extraction methods. An attempt to use the R fraction itself as an antigen in complement fixation tests for M failed because the R invariably reacted similarly to suspensions of CP. It was concluded that

remnants of CP on the inner surface of the fragments of hulls of streptococci were fixing complement with anti-CP antibodies in the serum. (All anti-M sera available had been prepared by injection of whole organisms.)

The Effect of Variation in the Time of Disintegration.—While studying the effect of varying the physical factors during fractionation, it was found that the yield of CP varied with the length of time of disintegration, whereas that of S remained approximately constant. Typical results of such experiments (Table VII) show that by increasing the time of sonic vibration it is possible to obtain increasing amounts of CP, at the expense of the yield of R. The gross chemical characteristics of the CP are apparently not different in the larger yields than in the smaller. The actual yields are affected by the efficiency of the vibration, which varies among runs, somewhat by the concentration of the organisms, and in at least one observed case, by the strain involved. In the case of strain H 44, the first hour of vibration releases more of the cytoplasmic particles than in the case of other strains, although the final partition of the contents of the streptococcal cell does not vary widely among the strains investigated.

Electron micrographs were taken of the products of sonic vibration of the streptococci. After an hour of vibration, the majority of the cells appear to have lost much of their contents and show partially empty hulls. A few of these hulls have begun to break up into crescentic fragments. After 4 hours of vibration there is marked fragmentation of the hulls of the organisms. Other data reported in this paper indicate that these bits of cell wall have been stripped of almost all the CP which adhered to them at first.

DISCUSSION

The Relation of the New Fractions to Fractions of Streptococcal Nucleoproteins in the Earlier Literature.—Although it is difficult to correlate the results of this physical fractionation with those of chemical extractions, it appears possible that the CP forms the major portion, or all, of the P nucleoprotein of Lancefield, the E-K group of Heidelberger *et al.*, and the NPA of Mudd and Lackman. There is little doubt that the "pigmented heavy particles" derived from sonic extracts of streptococci by Sevag, Smolens, and Stern are the same as the cytoplasmic particles described here. Several differences must, however, be noted. First, the CP described in this paper were white, with no trace of green pigment, except for one group of preparations which showed a faint gray-green cast. It may be that the green pigment of the macromolecules was the result of assimilation of a heavy metal by the streptococci. Again, the speed of centrifugation used in the earlier study to clear suspensions of the macromolecules of heavier impurities was in a range which we found to precipitate considerable amounts of the CP. Finally, the phosphorus content of CP as found in this study is lower than that noted by the earlier authors.

The question arises of the relation of the S fraction to Lancefield's Y and to

Heidelberger's D fraction. In the absence of specimens of those materials it is only possible to say the following: The Y component, which Lancefield encountered but could not isolate, was easily digested by trypsin; forthcoming experiments on tryptic digestion of S may show whether S could be the Y antigen. As for the D fraction of Heidelberger, there are two difficulties in an attempt to identify S with it: the apparently non-polar union of protein and nucleic acid in the D fraction and the fact that the E–K fraction absorbed out antibodies to D in antistreptococcal sera. Both of these findings would not be consistent with the characteristics of S.

The Relation of These Observations to the Total Antigenic Analysis of the Hemolytic Streptococcus.—The structure of the cytoplasmic particles makes it not impossible that antigenic groupings different from those on the surface might be "folded" in the interior of the particle and so not available for reaction *in vitro*. Since the physicochemical means used in resolving the S fraction are not applicable to the CP, other means are being used to investigate its immunologic homogeneity.

The Organization of the Streptococcal Cell.—As a result of much recent work with the constituents of cells other than bacterial, ribose nucleic acid has become associated with nucleoproteins of the cytoplasm and desoxyribose nucleic acid with nuclear elements (27, 28). In the work here reported it was found that the nucleic acid in the CP was predominantly of the ribose type, which is consistent with many observations on cytoplasmic particles derived from other cells. On the other hand, the nucleic acid in S gave the tests for desoxyribose nucleic acid so that it is likely that S contains nuclear material.

These considerations, as well as the inverse relationships between the yield of residue and that of CP, without change in the yield of S, suggest the following hypothesis: It is possible that at first rupture of the cell by the vibration there is fission of the cell wall and of the cytoplasm. The nuclear contents, which are not organized into complex units like those of the cytoplasm, and may be in a more liquid state, pour out in their entirety into the suspending medium. The cytoplasmic particles are then progressively shaken off the fragments of bacterial cells, to form an opalescent stable suspension. What would be called the insoluble residue after an hour of sonic vibration is in reality largely cytoplasmic material. Even after 4 hours of continuous vibration, not all of the cytoplasmic material has been shaken off the fragments of hulls or cell walls of the streptococci, and the inner surface of the latter is lined with cytoplasmic particles. In fact, the R fraction of 4 hour vibration was used twice with success as a substitute for CP in cross-absorption experiments. The truly insoluble residue, consisting of cell walls from whose inner surface all cytoplasmic particles have been stripped might be obtained only after long periods of vibration.

SUMMARY

After disintegration by sonic vibrations the contents of the hemolytic streptococcus can be separated by differential centrifugation into three fractions: an insoluble residue, cytoplasmic particles, and a solution of proteins of smaller unit size.

The residue (R) presumably comprises the cell walls of the bacteria and contains the type-specific M protein. The cytoplasmic particles (CP) contain some lipoid, the group-specific carbohydrate, and nucleoprotein of the ribose type. The supernate fraction (S) contains two components, presumably protein, at least one of which is part of a dissociable nucleoprotein of the desoxyribose type.

Both CP and S precipitate and fix complement with antistreptococcal sera. Both give rise to antibodies on injection into rabbits. Both are of broad reactivity. CP and S can be shown to be serologically distinct by several means, including cross-absorption tests.

On continued disintegration of the organism an inverse relation is noted between the yield of R and that of CP, whereas the yield of S is constant.

A theory as to the structure of the streptococcal cell is offered in terms of the data presented.

The author wishes to express his deep gratitude to Dr. Dan H. Moore, of the Electrophoresis Laboratory of the College of Physicians and Surgeons, New York, for carrying out and interpreting the electrophoretic and ultracentrifugal determinations used in this work.

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STUDIES IN THE RELATION OF THE HEMOLYTIC STREPTOCOCCUS TO RHEUMATIC FEVER

III. Complement Fixation versus Streptococcal Nucleoproteins with the Sera of Patients with Rheumatic Fever and Others*

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In the previous paper of this series (1) the isolation of two fractions of streptococcal nucleoproteins was described: the cytoplasmic particles (CP) and the S fraction, of smaller molecular size. It was pointed out that so far neither fraction had been prepared chemically free of the other. However, the immunologic reactions of each were so distinct and characteristic that the two fractions could be employed in serologic tests in their present state of purity. Although the S fraction was shown by electrophoresis and ultracentrifugation to contain two molecular species, presumably protein, the use of this preparation in exploratory serologic work is indicated by the facts that this fraction has not been described before, and that the relative concentrations of the two components of S are of the same order (33 and 67 per cent of their total, respectively).

Since antibodies to these fractions had never been measured, it was necessary to determine their titer in the normal population. Such tests were performed on the sera of newborn infants, older infants, children, and young adults. For evaluation in streptococcal infections tests were done on a group of patients with scarlet fever, serial specimens being taken at the acute and convalescent stages and several months thereafter. Finally, titrations were done on the sera of patients with active and quiescent rheumatic fever, and longitudinal studies were done during single or repeated rheumatic episodes. For general correlation with the earlier work on measurement of streptococcal antibodies, antistreptolysin tests were done on each serum tested.

Methods and Materials

The Preparation of Sera and Antigens.—The sera of newborn infants were prepared from blood collected from the umbilical cords in the Delivery Rooms of the Philadelphia General

* The work described in this paper was aided by a grant from the Life Insurance Medical Research Fund.

Hospital. The sera of older infants were collected at the Well-Baby Clinic of The Children's Hospital of Philadelphia. These infants ranged in age from 6 months to 2 years. The sera representing the antibody levels in normal children were those collected in the group of patients with scarlet fever mentioned below for the study of the serologic response in acute streptococcal infection. Only sera collected within the first 3 days of the disease were taken to represent normal antibody levels. The sera of young adults were collected at the Student Health Service of the University of Pennsylvania.

For the study of antibody response to these antigens in acute streptococcal disease, blood specimens were collected from children with scarlet fever at the Philadelphia Hospital for Contagious Diseases during the first half-week and during the 3rd week of the disease. In a number of these cases, an additional specimen was drawn 4 months later.

In the case of rheumatic patients the blood specimens were drawn at weekly, biweekly, or monthly intervals, depending on the severity of the rheumatic process, in correlation with the clinical studies to be described below.

Blood specimens were collected with a minimum of sodium citrate as anticoagulant, in order to allow for the determination of the erythrocyte sedimentation rate, white blood count, and concentration of hemoglobin. The plasma was drawn off each blood specimen and frozen as soon as the tests above had been completed. When a specimen was to be tested, it was thawed, and cleared by centrifugation of the fibrinogen which had precipitated on freezing. A single dilution of 1:8 or 1:16 was made of each serum, in sufficient amount to allow for the two complement fixation tests, the detection of anticomplementary sera, the antistreptolysin titer, and the antihyaluronidase titer (2). It was then heated to 56°C. for 15 minutes to inactivate complement. These dilutions were used within a few days, and were kept frozen between tests.

The S antigen was maintained in the refrigerator in the lyophilized state and was dissolved when needed. In the case of CP desiccation was not feasible, because of poor resolution of the large particles. The CP was maintained in suspension in the refrigerator with merthiolate added to a final concentration of 1:10,000. There were no troublesome anticomplementary effects during many months of such storage.

The Serologic Tests.—Complement fixation tests were performed by incubating 0.4 cc. of various dilutions of serum with 0.1 cc. of the antigen (at twice its minimal optimal concentration) and 0.1 cc. of complement, diluted to contain 1.3 to 1.5 units under the conditions of the test. After 45 minutes of incubation at 37°C., 0.1 cc. of a 4 per cent suspension of sheep erythrocytes was added to each tube, together with 0.1 cc. of rabbit anti-sheep–erythrocyte serum, adjusted to contain 2 units of hemolysin in that volume. The antigen-complement and the sensitized erythrocytes were each added as a mixture, using an automatic pipet. At the end of half an hour of further incubation at 37°C. the degree of hemolysis was read as 0, weak, strong, or complete. As a control on temperature effects in incubating, and because of the time required for the pipetting of reagents, at least one standard serum was included in each rack of every test. Each test with each antigen involved about 450 sera, including the standard sera. The usual precautions of complement titrations were taken, and several experimental sera of each test were repeated in the following one. Finally, for more precise comparison of titers in a given patient at different stages of activity of the rheumatic process, all sera of a given patient were included within one set to be tested.[1]

The antistreptolysin tests were done exactly as suggested by Todd (3), since that is the technic followed in almost all of the reported studies of antistreptolysin titers. Serum dilutions were made in volumes of 0.4 rather than 0.5 cc., in conformity with the other tests in this study.

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[1] The complement used in this study was the lyovac complement of Sharp and Dohme, Inc., to whom the author is indebted for generous gifts of this material.

The Study of the Patients and the Criteria of Activity of the Rheumatic Process.—The rheumatic patients were studied in acute, convalescent, and quiescent stages of the disease. They were usually seen first in the wards of The Children's Hospital of Philadelphia, or the Philadelphia General Hospital. Many of them were studied in the wards of the Children's Seashore House at Atlantic City during the chronic, active and the convalescent stages of the illness. They were then followed at the Rheumatic Fever Clinics of the first named institutions in the quiescent stage. In some cases, of course, the entire cycle was repeated.

The patients were examined at least every 2 weeks while in the acute or convalescent wards and at each visit to the Clinics. These examinations included the following: symptoms—anorexia, headache, precordial, abdominal, or arthritic pain, cough, dyspnea, and epistaxis; physical signs—cardiac rate and rhythm, distance of apex beat from the midline, murmurs, with distance of transmission of each, and other adventitious sounds, palpable thrills and friction rubs, hepatic enlargement and edema, rashes, subcutaneous nodules, and chorea. Laboratory examinations included the erythrocyte sedimentation rate, white blood cell count, and hemoglobin concentration. The vital capacity was usually determined at biweekly intervals, and electrocardiograms were taken as required. The erythrocyte sedimentation rate was done by a method described elsewhere (4). It involved a series of readings of the erythrocyte level at 5 minute intervals, in order to determine the rate of free fall of the corpuscles, and a correction for the relative volume of erythrocytes.

The patients selected for serologic study were, of course, only those in whom the diagnosis of active rheumatic fever was beyond doubt. Very nearly all of these children had active rheumatic carditis.

The recording of clinical evidence of rheumatic activity in correlation with the serologic titers presented a problem. A considerable part of the clinical data was kept in graphic form in the records of this investigation. However, an attempt to present all such data in graphic form in this paper was abandoned because of the necessary complexity of such graphs. On the other hand, the manifestations of active rheumatic disease are so protean that no one or two clinical signs can measure the activity in all cases. The erythrocyte sedimentation rate is probably the most sensitive single indicator of activity of the rheumatic process, but it has been shown that this test may be unreliable in adolescent and overweight children (4), in right-sided heart failure (5, 6), and in prolonged, full dosage of salicylates (7–9). Finally, there are some patients in every series of sufficient size whose erythrocyte sedimentation rate is within normal limits, although there is unquestionable clinical evidence of an active rheumatic process.

For the recording of the clinical evidence of the degree of rheumatic fever, therefore, it was decided to show the erythrocyte sedimentation rate, temperature, resting pulse rate, and such episodes as polyarthritis, carditis, and cardiac failure. The criteria of quiescence of the rheumatic process included the fall to a plateau level of the erythrocyte sedimentation rate, of the size and rate of the heart, and of murmurs and their extent of transmission; a stable pulse rate, absence of significant symptoms and signs, and general well being.

RESULTS

The Optimal Antigen Concentration of the Fractions.—In order to use the two nucleoprotein fractions in large scale serologic tests it was necessary to ascertain that the optimal range of concentration of each fraction as antigen in complement fixation tests was the same, regardless of the titer of the antiserum. Table I shows that such is the case for high and low titered sera of rheumatic patients. The same optimal concentrations were found to obtain also in tests using rabbit antistreptococcal sera of high and low titer.

The Antibody Levels to CP and S in Normal Subjects of Various Age Groups.— Since nothing was known of the occurrence of these antibodies in human sera, determinations were carried out of the complement fixation titers to CP and S in sera of 35 neonatal infants, of 35 older infants ranging from 6 months to 2 years, of 70 children ranging from 4 to 15 years, and of 40 medical students. Comparative tests of antihemolysin were done in parallel. The results of these tests are shown in the form of a percentage frequency chart in Fig. 1.

Fig. 1 shows that antibodies to all three streptococcal antigens under investigation are found in the sera of normal subjects. Although the range of titers

TABLE I

The Constancy of Optimal Antigen Titers of CP and S in Complement Fixation Tests versus Rheumatic Sera of High and Low Titer

	Serum 1315 (active rheumatic)							Serum 1910 (quiescent rheumatic)			
	Dilution 1:							Dilution 1:			
	32	64	128	256	512	1024	2048	8	16	32	64
CP											
0.01 per cent solution	0	0	0	0	0	tr	s	0	w	s	c
0.005	0	0	0	0	0	tr	s	0	w	s	c
0.0025	0	0	0	0	0	0	s	0	w	c	c
0.0013	w	w	w	w	s	s	s	w	s	c	c
S											
0.2 per cent solution	0	0	w	s	c	c	c	0	s	c	c
0.1	0	0	0	s	c	c	c	0	w	c	c
0.05	0	0	tr	s	c	c	c	0	w	c	c
0.025	0	tr	s	c	c	c	c	w	s	c	c
0.013	w	s	c	c	c	c	c	ac	c	c	c

The degree of hemolysis is recorded: O, none; tr, a trace; w, weak; s, strong; ac, almost complete; c, complete.

differs among the three tests, certain broad similarities are seen. In each test, a considerable percentage of relatively high titers is found in neonatal infants. After several months, or a year, the higher titers are no longer found except in a very few cases. In childhood higher titers appear in all three tests, and among young adults the higher titers occur with slightly greater frequency than among children.

The Antibody Response in Acute Streptococcal Disease.—

Since no epidemic of streptococcal infection occurred in the Philadelphia area during the course of these studies, the patients chosen for examining antibody levels in streptococcal infection were children with scarlet fever. These children were admitted to the Philadelphia Hospital for Contagious Diseases within the first day or two of the disease. The diagnosis was verified at the hospital, and the children were not treated with sulfonamides. Sera drawn at

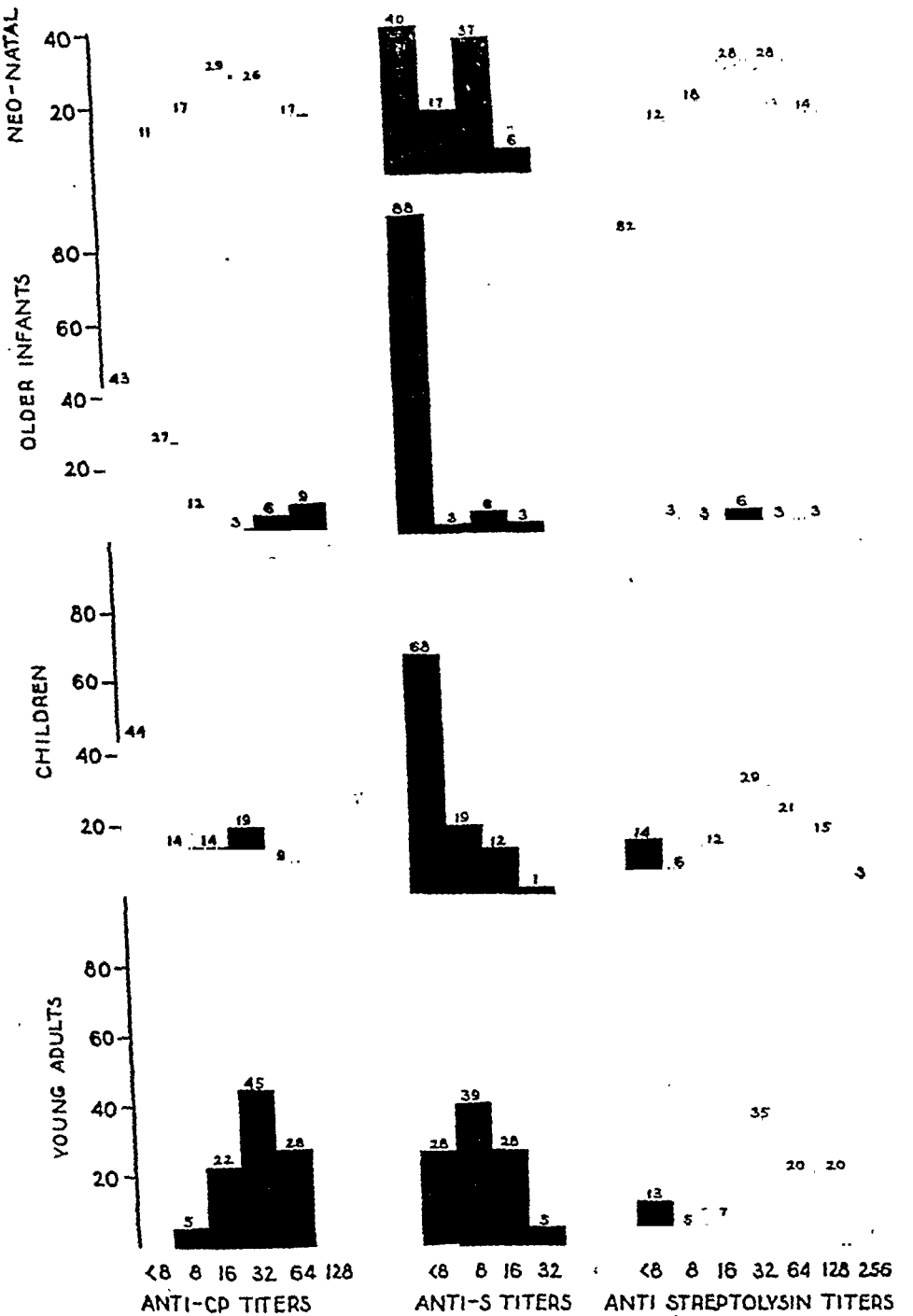


FIG. 1. The frequency distribution, in percentage, of complement fixation titers to CP and S. and of antistreptolysin titers, in sera of normal subjects of various age groups.

the beginning and at the end of the 3 week hospitalization were compared to determine increases in antibodies in the course of the infection. As can be seen by the distribution of normal titers in Fig. 1, the titers at the first bleeding varied widely. Accordingly, the effect of the disease on the antibody level is recorded, in Table II, in terms of individual increases, rather than of average titers.

Table II shows that in all the tests 80 per cent or more of the patients showed at least a twofold rise in titer, so that an increase of antibody level in any of these three tests may be considered characteristic of this acute streptococcal infection. There was poor correlation among the changes of antibody titers to the respective antigens. Although a few sera showed very little or no rise in the titer of all three antibodies, there were examples of sera in which one of the antibodies showed a greater increase in titer than in the case of the other two. This could be

TABLE II

Percentage Frequency of Increases in Antibody Titers to CP, S, and Hemolysin in the First 3 Weeks of a Group of 70 Cases of Scarlet Fever

Increase in titer	CP	S	ASL
None or too small to be significant	20	15	17
2-fold	39	66	37
4-fold	22	17	11
8-fold	14	2	8
16-fold	4	0	7
32-fold or higher	1	0	20

observed for any of the three antibodies, especially in the case of anti-CP and antihemolysin, in which the range of increase tended to be higher.

In order to study the persistence of these antibodies after recovery from the streptococcal infection, sera were obtained from these patients 4 months after they were discharged from the hospital. Twenty such specimens were obtained. Comparison of the titers of these sera with those of the specimens drawn at 3 weeks showed that the majority of titers to each of the antigens remained elevated for at least that period of time. In the case of the CP, 70 per cent of the pairs of sera showed no significant change in titer. In 10 per cent, there was a twofold fall in titer, and in 20 per cent, there was a twofold rise. Anti-S antibodies showed as high a titer after 4 months in 80 per cent, a fall in 10 per cent, and a rise in 10 per cent. The antistreptolysin titers were unchanged in 65 per cent of the pairs tested. The 4-month specimens showed higher titers in 10 per cent of the pairs, and slightly lower titers in the remaining 25 per cent. In general the titer of all three antibodies was in the same range after 4 months of health as during the preceding episode of scarlet fever.

Antibody Titers in Acute and Quiescent Rheumatic Fever.—The sera of 100

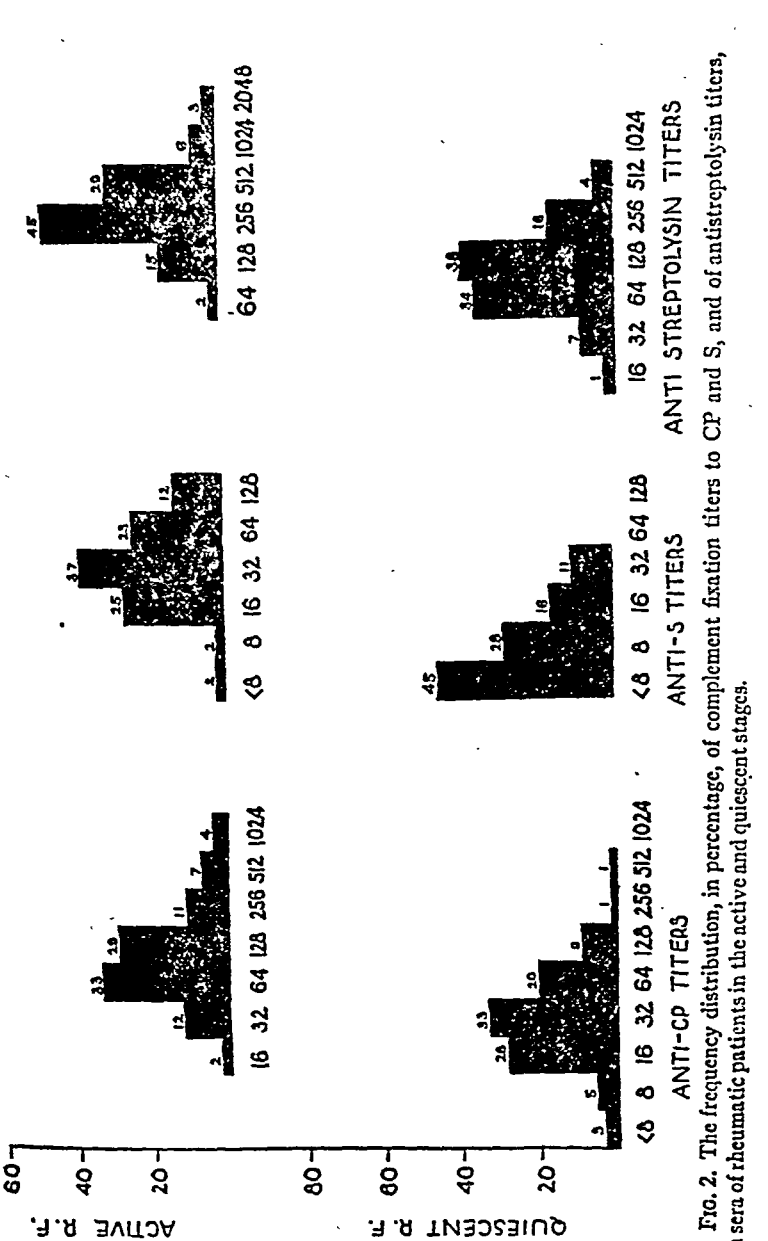


FIG. 2. The frequency distribution, in percentage, of complement fixation titers to CP and S, and of antistreptolysin titers, in sera of rheumatic patients in the active and quiescent stages.

children with active rheumatic fever were examined for antibodies to these three streptococcal antigens, as well as the sera of 81 rheumatic patients in the quiescent stage of the disease. The frequency with which various titers were found is shown in percentages in Fig. 2.

Fig. 2 shows that the antibodies in all three tests were elevated in the great majority of active cases, in comparison with those of normal children shown in Fig. 1. In the case of quiescent rheumatic patients fewer elevated titers are found, but there are more of these than among the normal individuals.

A comparison of the distribution of titers in active rheumatic patients with those of normal controls of the same age group suggests a titer of 32 as a dividing line between the anti-CP titers of normal children and active rheumatic patients, 8 for the anti-S titers, and 128 as the analogous antistreptolysin level. The significance of these levels and of the difference between titers of normal and acute rheumatic children is brought out in Table III.

TABLE III

Titers of Normal, Active Rheumatic, and Quiescent Rheumatic Children against CP, S, and Hemolysin, in Percentages

	CP		S		ASL	
	Up to 32	64 and above	Up to 8	16 and above	Up to 128	256 and above
Normal children	91	9	87	13	97	3
Active rheumatic fever	13	87	4	96	17	83
Quiescent rheumatic fever	69	31	73	27	80	20

Table III shows that a range of anti-CP titers exists which includes so high a percentage of normal children that, for streptococcal work, it can be considered the upper normal limit, and that the great majority of active rheumatic subjects have titers above that level. The same is true of anti-S titers. The analogous results with antistreptolysin titers confirm those recorded in the literature. The sera of quiescent rheumatic patients show a much smaller number of titers above the upper normal limit, but more than in the case of normal individuals. The average intervals between the foregoing acute rheumatic episode and the time of collection of serum from these quiescent rheumatic patients were 17 months.

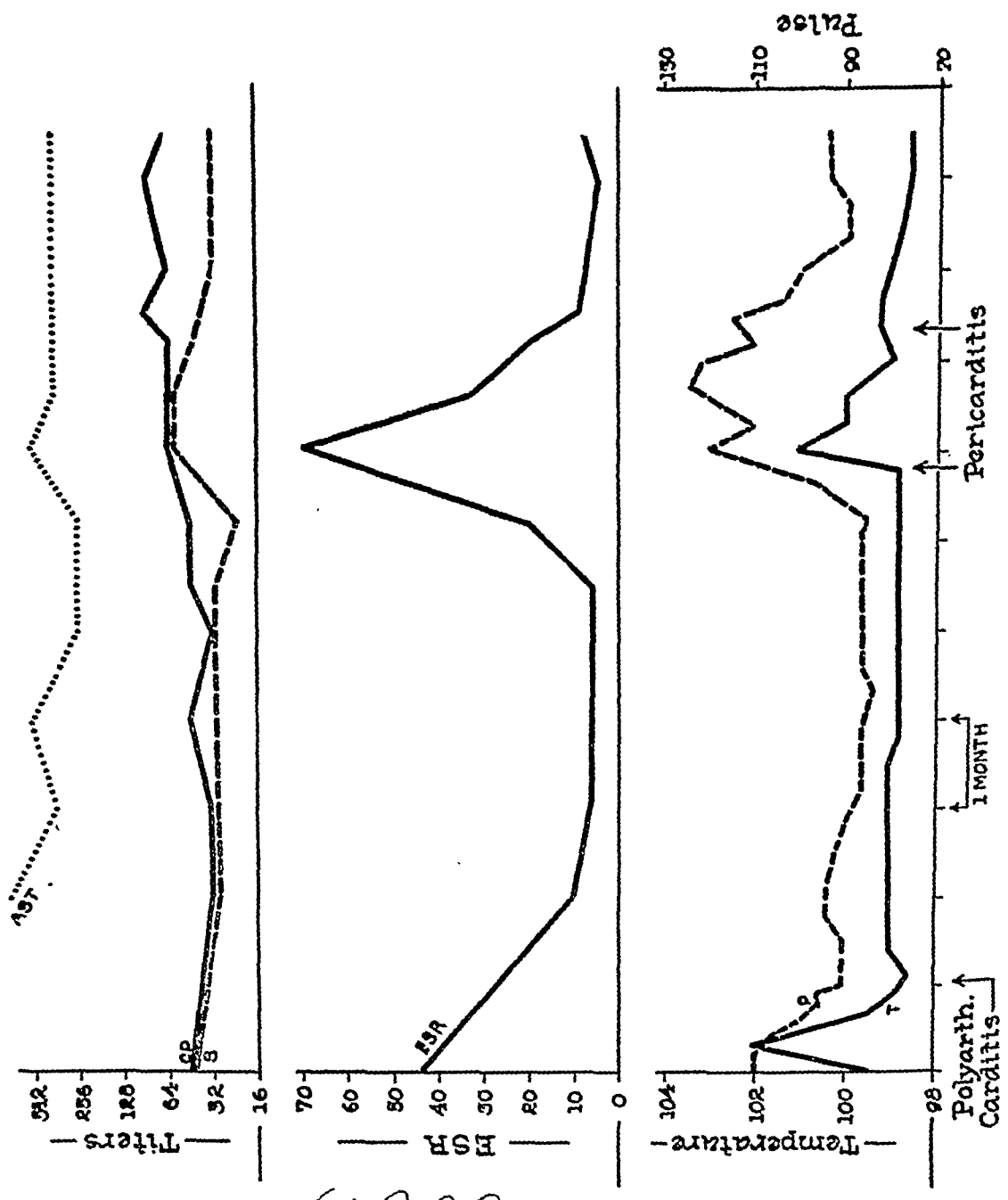
Antibody Titers during Fluctuations in the Severity of the Rheumatic Process.— The great majority of the patients with acute rheumatic fever were followed into the stage of quiescence. A few had continuous rheumatic activity either of fulminating severity ending in death, or of a low grade. A number, however, ran a classical polycyclic course with clearly defined recurrences or changes in clinical estimate of rheumatic activity. In the single rheumatic episodes

studied the titers of all three antibodies did not decline as the clinical activity of the disease subsided, but remained elevated for months after complete quiescence of the rheumatic process, in the absence of any clinical streptococcal infection. The patients with a polycyclic course afforded an opportunity to correlate antibody titers with variations in degree of rheumatic activity. The charts of three such patients, shown in Fig. 3, reveal that, although the titers vary during the course of the rheumatic infection, there is no correlation with the clinical changes used to evaluate the severity of rheumatic fever. In none of the cases shown had sufficient time elapsed between recurrences for the titers to fall to normal levels.

DISCUSSION

The presence of the antibodies measured in these studies in normal sera, and their concentrations at various age groups, are a consequence of the prevalence of the hemolytic streptococcus in the human throat and of the breadth of specificity of the antigens. After several months or a year the infant has lost the streptococcal antibodies derived from the maternal blood; the titers are quite low at that age. In childhood, however, as opportunity for contact with the ubiquitous streptococcus increases, higher titers appear and these are again slightly higher in young adults. Among the antistreptolysin values, the comparison of titers between neonatal life, later infancy, and childhood is in good agreement with that reported for that test by Gordon and Janney (10).

The prevalence of the hemolytic streptococcus and the broad specificity of these antigens are also the basis for the prolonged maintenance of elevated titers after acute streptococcal or rheumatic disease. In this case it is probable that minute amounts of antigens, produced by streptococci too few in number to excite an inflammatory reaction, suffice to act as "boosters" to maintain or raise a titer already elevated by disease. In the case of both streptococcal and rheumatic infection, Mote and Jones (11) have shown that antistreptolysin titers may remain elevated for as long a period as 1 year, without clinical infection by the streptococcus. Solomon (12) has shown the same effect in streptococcal infection, and Yannet and Leibovitz (13) had similar results with antifibrinolysin in streptococcal disease. In the studies reported here anti-CP and anti-S titers were followed for 4 months after streptococcal infection. At the end of that time the average titers were the same as at the height of the disease. Following acute rheumatic infections there was a better opportunity to observe the rate of fall of the antibodies to CP and S. These antibody titers could remain elevated for many months after the evidence of rheumatic activity had subsided. It is very likely that the greater percentage of elevated titers in the quiescent rheumatic group than among normal controls was due to the same phenomenon as that which keeps these titers above normal after streptococcal infection, and not to any characteristic reaction of the quiescent rheumatic patient to these antigens.


Titers
512
256
128
64
32
16
GP
S
ASO
ESR
70
60
50
40
30
20
10
0
RSR
Temperature
104
102
100
98
Pulse
130
110
90
70
Polyarth.
Carditis
1 MONTH
Pericarditis

61888

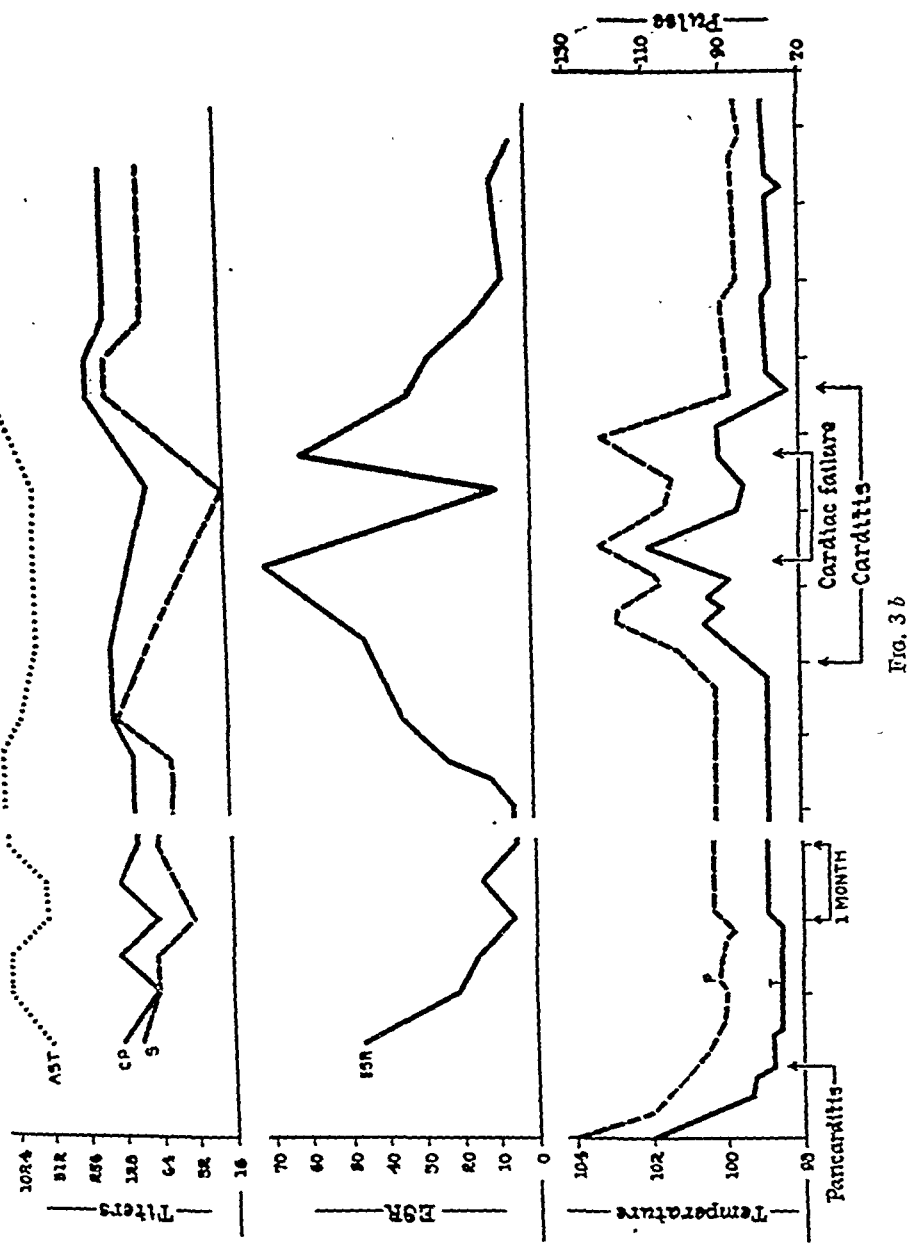

Titers
1024
512
256
128
64
32
16
AST
CF
S
ESR
70
60
50
40
30
20
10
0
ESR
Temperature
105
102
100
98
Pancarditis
1 MONTH
Cardiac failure
Carditis
Pulse
130
110
90
70

FIG. 3 *b*

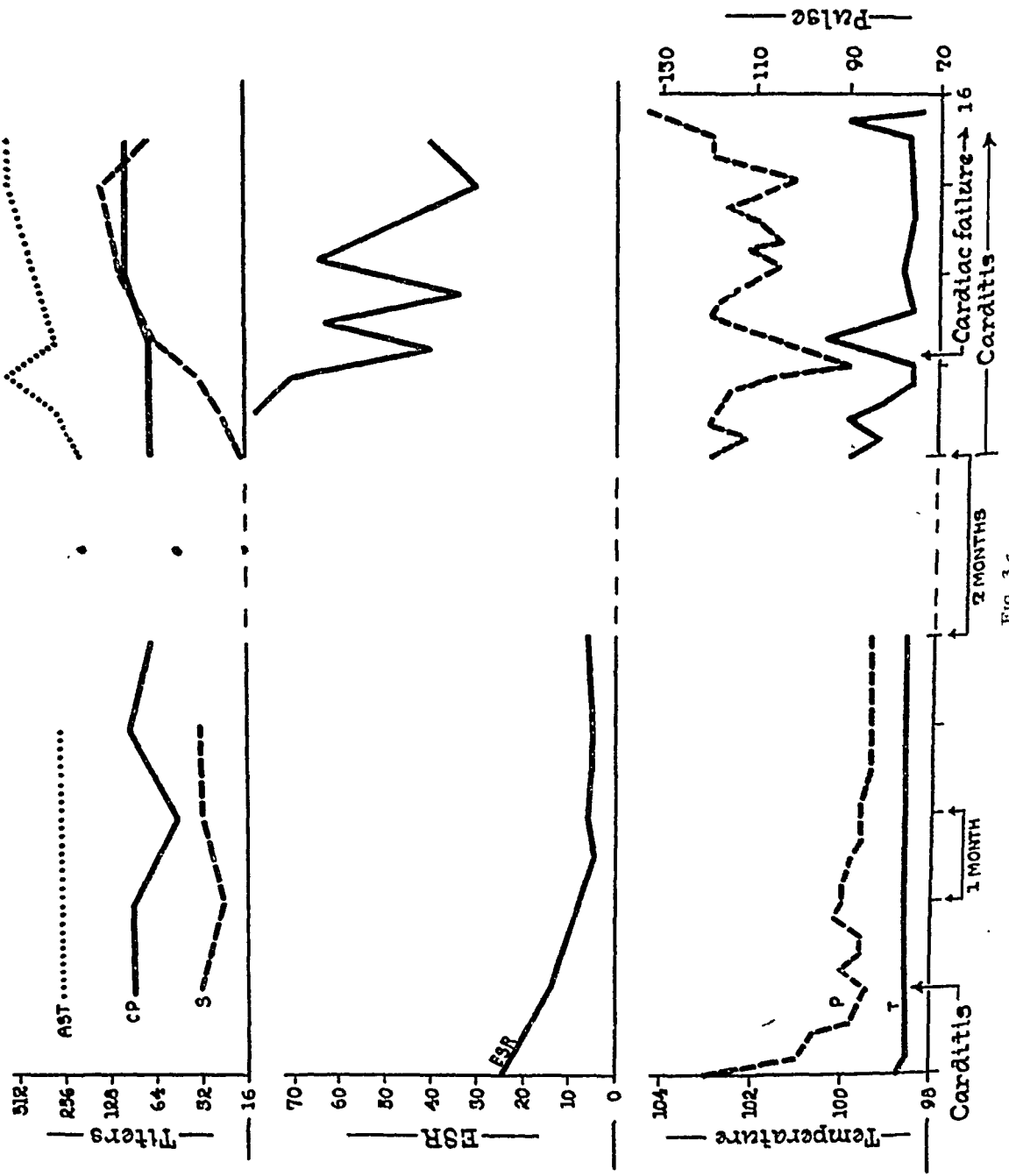

Titers
512
256
128
64
32
16
AST
CP
S
ESR
70
60
50
40
30
20
10
0
ESR
Temperature
104
102
100
98
T
P
Pulse
130
110
90
70
Carditis
1 MONTH
2 MONTHS
Cardiac failure
16
Carditis

FIG. 3 c

The factors which account for the prevalence and persistence of these antibodies also make it necessary to assume an upper limit of normal titers which does not actually include the highest titers among normal subjects. As striking as is the contrast in distribution of titers between active rheumatics and controls, it would be impossible to demonstrate it if one stipulated that the upper limit accepted for the normal population include all the titers found in that group. The epidemiology of the streptococcus makes it necessary to set the upper limit of normal at a titer which includes only 85 or 90 per cent of normal sera tested. Even without accepting the levels indicated in Table III as upper limits of normal for the respective antibody titers, the difference in distribution of titers between normal subjects and active rheumatics is striking, and statistically significant. The serologic tests with CP and S confirm the suggestion derived from antifibrinolysin and antistreptolysin tests that the active rheumatic patient is one in whom streptococci are present in numbers comparable to those in an acute streptococcal infection.

It is not possible to be certain at present whether either of the new tests is preferable to the antistreptolysin test for general use. The anti-S titers show the greatest difference between the acute and quiescent phase groups. Whether this difference is sufficiently great to indicate the use of anti-S complement fixation for semiroutine work will be decided as additional serologic tests are done.

The question inevitably arises as to the rôle of CP and S in the pathogenesis of rheumatic fever. It should be pointed out that if the pathogenesis of rheumatic fever involves sensitization, it is possible that the sensitizing agent will not be detected by serologic tests, because there is no necessary quantitative correlation between the degree of sensitization and the titer of humoral antibodies to a given substance. Especially in the case of streptococcal immunology, the disturbing factor of prevalence of subclinical contacts with the organism would make the serologic identification of a streptococcal sensitizing agent very unlikely, should such an agent exist in rheumatic fever. The work presented here should not be regarded, therefore, as an examination of the CP and S as possible pathogenetic agents in rheumatic fever, but rather as a serologic exploration of sera of normal, streptococcal, and rheumatic subjects by these new antigens.

SUMMARY

Complement-fixing antibodies to the cytoplasmic particles (CP) and to the S fraction of streptococcal nucleoproteins are present in normal human sera, the range of concentrations varying among the age groups.

The titer of these antibodies rises between the first half-week and the 3rd week of scarlet fever, in more than 80 per cent of the cases. The titers then remain elevated for at least 4 months.

In children, 91 per cent of the normal sera examined showed anti-CP titers up to 32; 87 per cent of sera in active rheumatic disease had titers above this level. Corresponding data with S fell in the same range of percentage distribution.

Anti-CP and anti-S titers remained elevated long after the rheumatic process had reached quiescence. No correlation of serologic titer with the degree of clinical activity was found in the case of either antibody.

The author wishes to thank Dr. A. C. LaBoccetta, of the Philadelphia Hospital for Contagious Diseases, for permission to obtain the serum specimens of the patients with scarlet fever, and Dr. Werner Henle, of The Children's Hospital of Philadelphia, for the serum specimens of older infants and young adults.

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HEPARINEMIA (?)*

An Anticoagulant in the Blood of Dogs with Hemorrhagic Tendency after Total Body Exposure to Roentgen Rays

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Plates 1 and 2

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Hemorrhage is one of the important abnormalities occurring in animals and man exposed to large doses of ionizing radiation delivered over the entire body. Fernau, Schrank, and Zarsicki (1) as early as 1913, reported hemorrhage in rabbits following the injection of one hundred or more electrostatic units of polonium. Death usually occurred before the 12th day, at which time the blood was incoagulable. These observations have been confirmed by Falta (2) and others (3, 4). In these reports, hemorrhages were extensive and all organs of the body were affected. It was suggested that the associated thrombocytopenia was the probable cause of the hemorrhagic state.

In the dog exposed to x-irradiation of the entire body many pathologic alterations may occur. The most obvious of these are hemorrhage and infection. Hemorrhage is capable in itself of killing the animal, and it may appear as a result of exposure to most forms of ionizing irradiation (1–6).

The Irradiation Syndrome in Dogs

The data comprising this report were obtained from studies on dogs given an x-ray exposure of 450 roentgen units over the entire body. Daily observations were made on each animal whenever possible, including the whole blood clotting times, the prothrombin time, clot retraction, erythrocyte count, leukocyte count, differential leukocyte count, platelet count, hematocrit reading, and sedimentation rate. Daily recordings of the rectal temperatures were made and the physical condition of each animal was noted. The earliest signs of bleeding were usually observed at the points of greatest trauma, especially at the point of needle puncture. Hemorrhages in the mouth, the soles of the feet, and the subcutaneous tissue on the sides of the animal usually appeared first, generally during the 2nd week. The hemorrhagic state progressed and there were spontaneous hemorrhages from the rectum, vagina, and urinary tract. At death, 2 or 3 days later, hemorrhages were the chief findings. They were abundant throughout the gastrointestinal tract and were most marked in the colon and proximal four-fifths of the stomach. They occurred throughout the small bowel but were more prominent in the duodenum and lower ileum. They were almost invariably present in

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the heart muscle where blood was extravasated along the course of the coronary vessels. The lungs, parietal pleura, and diaphragm frequently showed extensive subserous hemorrhages, and they were occasionally seen in the gall bladder, urinary bladder, and skeletal muscles. The lymph nodes throughout the entire body appeared swollen, hemorrhagic, and soft. Although gross evidence of hemorrhage was not generally present in the solid organs, microscopic evidence of hemorrhage was almost always found. In general those organs subject to motion displayed the most marked evidence of hemorrhage.

TABLE I

The average clotting time, hemocytologic findings, hematocrit reading, and sedimentation rates are recorded for 25 dogs, exposed to 450 r over the entire body and untreated thereafter. The findings as given were complicated by occasional blood transfusions.

Days	Clotting time	Platelet count	Blood counts		Hemoglobin	Hematocrit reading	Sedimentation rate (60 min.)	Smear					Body temperature	Day of first hemorrhage	Death
			RBC	WBC				Neutrophiles	Lymphocytes	Mononuclears	Eosinophiles	Basophiles			
	min.	*c.mm.*	*c.mm.*	*c.mm.*	*gm. per cent*	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*°F.*		
Control	11.7	300,000	5.94	12,432	14.6	48	7.06	57.375	39.75	.05	2.1250	0.125	102.0		
1st	18	247,000	5.47	8,182	13.8	46.8	10.7	69.2	27	1.4	2.4	0	101.85		
2nd	17	211,200	6.13	5,760	13.7	44	19.8	75.6	20.4	0.6	2.4	0	101.85		
3rd	29	210,400	6.07	6,892	14.4	45	12.1	78.67	17.083	0.083	4.083	0.083	101.9		
4th	28	114,800	5.23	3,000	12.8	42.4	14.6	51.17	43	0.33	0.17	0	101.73	2 dogs	
5th	36	122,500	5.08	2,205	12.0	37.9	12.7	48.25	43.675	0.75	0.125	0	102.2	3 "	
6th	30	133,111	5.124	1,484	12.1	38.6	27.75	40.7	57.7	0.8	0.8	0	103.0	4 "	2 dogs
7th	35	111,583	4.74	504	10.7	36.0	26.2	30.4	57.6	2.0	0	0	103.2	4 "	1 "
8th	36	92,145	4.93	461	10.9	35.2	47.0	23.4	75.4	1.1	0	0.1	103.7	3 "	1 "
9th	49.49	78,500	4.316	702.9	13.37	33.4	47.64	26	74	0	0	0	104.6		3 "
10th	33.3	91,700	3.94	175	9.45	30.5	56.5	15	84	1	0	0	105.6	3 "	4 "
11th	41.15	52,923	3.566	292.45	9.84	31.2	59.5	10	90	—	—	—	104.98	3 "	3 "
12th	—	—	—	—	—	—	—	—	—	—	—	—			5 "
13th	81.7	34,167	—	433.3	10.13	—	—	—	—	—	—	—			
14th	—	—	—	—	—	—	—	—	—	—	—	—		3 "	2 "
15th	93.3	20,000	—	—	—	—	—	—	—	—	—	—			4 "

Many animals displayed bleeding ulcers in the mouth, duodenum, and especially in the colon. These hemorrhages were frequently complicated by infection.

The remainder of the picture displayed by dogs was that of an acute intoxication. They became listless and failed to eat. Fever developed, the sedimentation rate was increased, leucopenia and thrombocytopenia appeared, and frequently bacteria could be found in the terminal blood smear. Death usually occurred between the 9th and the 12th days. The hemorrhagic state was characterized by a prolonged clotting time which was easily studied.

The picture displayed by these untreated dogs was almost uniform, although the survival times varied from 7 to 15 days with an average of 11 days. The hematologic findings and clotting results for all untreated animals were averaged and are presented in Table I. To shorten this table, some data have been omitted.

EXPERIMENTAL

The technique used in the exposure was fairly constant. The source of x-rays was a 200 K.V.P. machine. The rays were filtered with 0.5 mm. of copper and 1 mm. of aluminum. The tube was operated at 15 milliamperes. The dosage rate was 6 r per minute as measured in air at a distance correspondng to the center of the animal's body and was checked before each exposure. This method has been described by Hagen and Zirkle (7).

The clotting studies made included the prothrombin time (8), the whole blood clotting time (9), and a heparin titration test carried out on whole blood. This last procedure will be described later.

Because the course and laboratory findings displayed by the first animal, dog 1-08, did much to pattern the subsequent experiments, the observations made on this animal are presented as representative of the group.

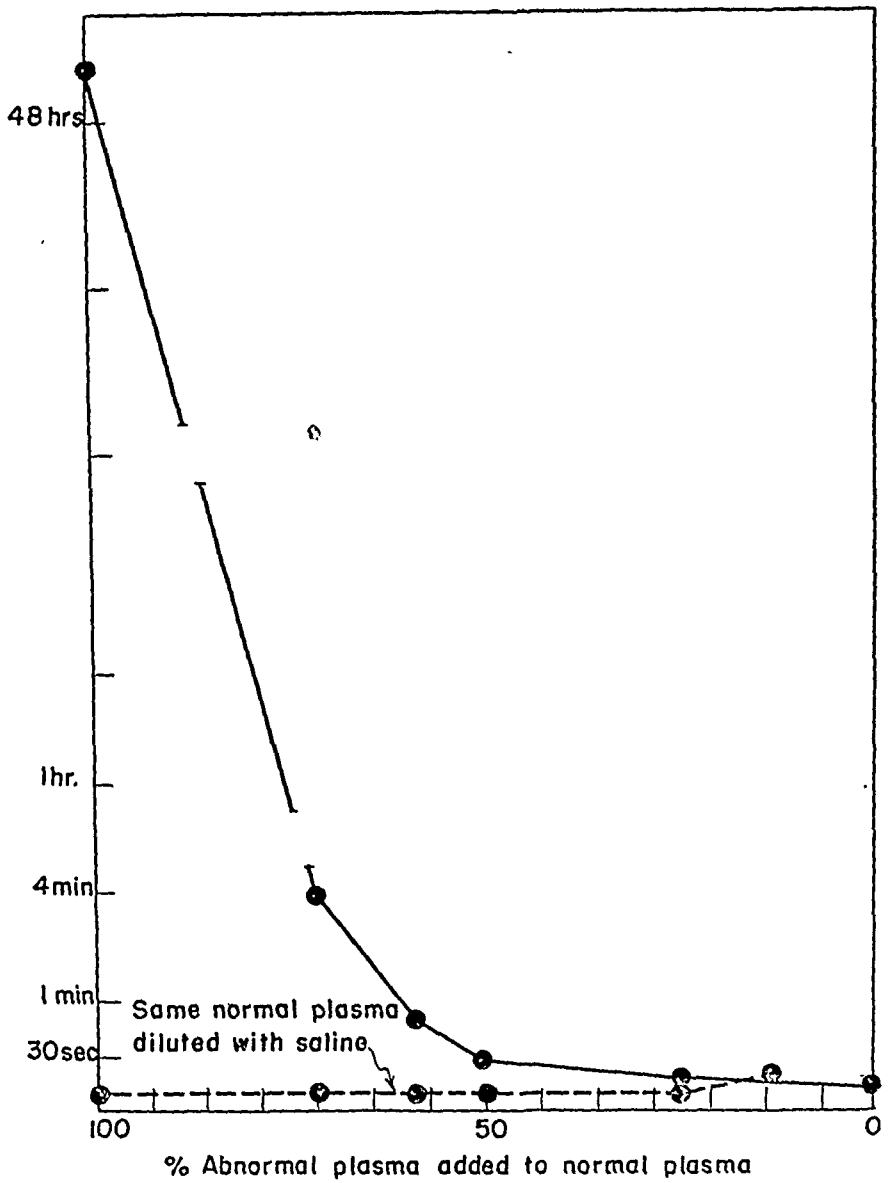
Dog 1-08, male, weighing 9.7 kilos remained in apparent good health for the first 10 days after x-ray exposure. The clotting time, however, was markedly prolonged after the 6th day, and on the 9th day there was bleeding from the mucous membranes of the mouth and rectum. The bleeding time was also prolonged and the animal bled freely from all points of needle puncture even though some of these punctures had been made several days previously. On the 9th day the dog's blood failed to clot and remained fluid in the test tube even after it became hemolyzed 2 days later. It could not be made to clot by adding thromboplastin, so the ordinary prothrombin time could not be determined. The platelet count on the 9th day was 260,000 and therefore did not seem to account for the bleeding. The blood did clot, however, when trypsin, papain, or purified thrombin was added. Since prothrombin is necessary for trypsin to clot fibrinogen, and since papain or thrombin clot fibrinogen independent of prothrombin, these findings demonstrated that both prothrombin and fibrinogen were present.

(*a*) *Demonstration of an Anticoagulant.*—

The failure of the blood to clot except when the above mentioned substances were added, even though thromboplastin, fibrinogen, prothrombin, and calcium were present, suggested that its incoagulable state might be due to the presence of an anticoagulant. This phase of the problem was next explored.

The plasma of dog 1-08 was mixed at various concentrations with oxalated normal dog plasma. The clotting times of these mixtures were then determined after the addition of thromboplastin and calcium chloride. Under these conditions the clotting times of the normal plasma were prolonged. For example, when the mixture contained 25 per cent normal plasma and 75 per cent plasma from dog 1-08, the clotting time was 4 minutes. In contrast, when a mixture of 25 per cent normal plasma and 75 per cent normal saline was used, the clotting time was 20 seconds. The observations were repeated, using separately various amounts of abnormal plasma (dog 1-08) and similar quantities of saline, and the effects of each upon the clotting times of normal plasma observed. The results are shown graphically in Text-fig. 1. These findings indicated that the delayed clotting times of the normal plasma-abnormal plasma mixtures were due to an active inhibition, not to mere dilution of

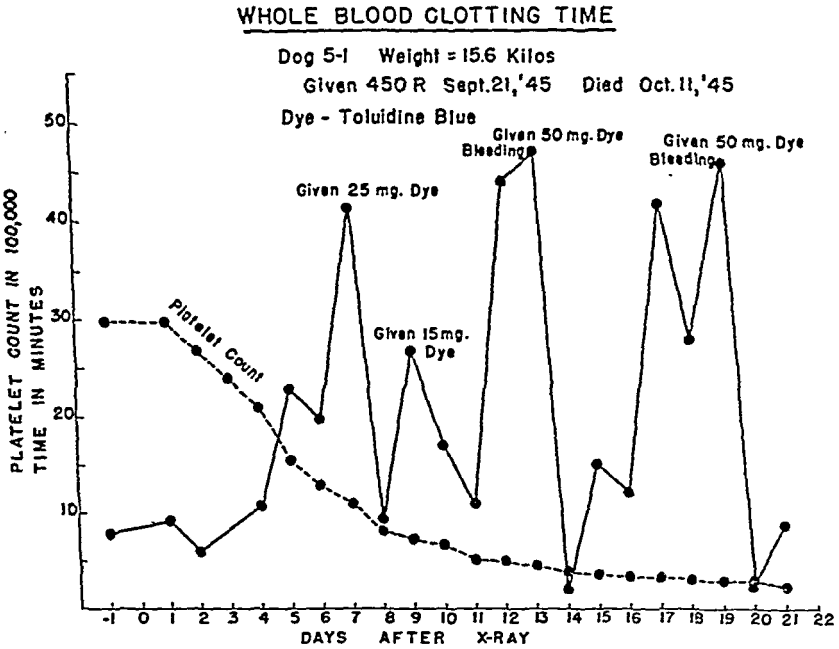
normal plasma by an inactive plasma. It was presumed that the abnormal plasma contained an anticoagulant.



TEXT-FIG. 1. The inhibitory effect of the plasma of dog receiving 450 r on the clotting of normal plasma.

In view of these findings, it seemed worth while to examine the abnormal plasma for the presence of heparin, the only known, naturally occurring endogenous mammalian anticoagulant. Heparin, an acid substance, can be bound and biologicaly inactivated by certain basic dyes and proteins (10, 11). Protamine sulfate and

toluidine blue are both active in this respect and can be used to exclude heparin from the clotting system by the addition of just enough of either to overcome the anticoagulant properties of heparin. These tests were made with the plasma of dog 1-08. Standard volumes of oxalated plasma with various amounts of toluidine blue or protamine sulfate were incubated for 20 minutes, and then beef lung thromboplastin and calcium chloride were added and the clotting times determined. Both of these substances proved capable of returning the clotting time to normal from its previously



TEXT-FIG 2. Demonstration of the effect of toluidine blue to return the prolonged clotting time to normal. The curve is characteristic of the phenomenon in other dogs which had received 450 r.

incoagulable state. The results are recorded in Text-fig. 2. The results of these two experiments suggest that some of the biochemical properties of the anticoagulant were similar to those of heparin.

The amount of toluidine blue necessary to restore the normal clotting time in dog 1-08 was calculated on the basis of these *in vitro* studies, after estimating the plasma volume; and this amount (25 mg.) was injected intravenously. Within 20 minutes all external bleeding stopped. Blood drawn at this time clotted within 4 minutes, while three separate samples, each distributed in a series of ten tubes, drawn just before injection remained fluid until they were discarded 2 days later. The blood from the dog was sampled on five more occasions during the 12 hour period following the injection, and on each occasion the clotting time was normal. Subsequently, it was observed

that gastrointestinal hemorrhages were always present in the untreated animals, and that these could also be prevented by the administration of toluidine blue or protamine sulfate (Figs. 1 and 2).

Both toluidine blue and protamine sulfate proved anticoagulant in themselves, when used in excess. Their anticoagulant properties are shown in Tables II and

TABLE II

Effect of Various Quantities of Toluidine Blue on the "Prothrombin Time" of the Plasma of Dog 1-08

Toluidine blue	"Prothrombin time"	Protamine sulfate	"Prothrombin time"
mg./0.2 cc. of plasma	*sec.*	*mg./0.2 cc. of plasma*	*sec.*
0.00	Incoagulable	0.00	Incoagulable
0.020	110	0.020	100
0.040	85	0.040	65
0.060	70	0.060	60
0.080	300	0.080	480
0.160	12 hrs.	0.160	12 hrs. approximately
0.225	20 "	0.225	20 " "

TABLE III

The Anticoagulant Effect of Toluidine Blue

Toluidine blue	"Prothrombin time"
mg. per cc. of plasma	*sec.*
0.02	28
0.018	26
0.016	24
0.014	23
0.012	23
0.010	22
0.008	20
0.006	20
0.004	19
0.002	20
0.000	20

III. However, the amounts necessary to prevent clotting were considerably more than that required to establish normal clotting in dog 1-08, as was also the case in other dogs subsequently tested.

(*b*) *Observations on Other Factors Concerned with Coagulation.*—Once it was possible to cause the plasma to clot, examinations for other plasma or blood deficiencies relevant to coagulation were made. The factors examined included: plasma prothrombn, serum calcium, plasma fibrinogen, and the platelets.

1. Plasma Prothrombin: The prothrombin time was determined on the plasma of all the irradiated dogs. Except in the terminal state, it was found normal if the plasma was first incubated with appropriate amounts of toluidine blue or protamine sulfate. The latter proved more uniformly active, and save for the first few experiments, protamine sulfate was the agent used. When plasma was not first incubated with protamine sulfate, the "prothrombin time" was occasionally prolonged by the anticoagulant present.

Vitamin K was administered to a group of irradiated dogs and was found to be ineffective in preventing hemorrhage. Data from four of these dogs are presented in Table IV. These animals received intravenously 5 mg. per day of an active vitamin K preparation (synkayvite-Roche) throughout the post-irradiation period. Three of these animals showed the usual prolonged clotting times which appeared 1 to 2 days before death. At autopsy hemorrhages were widespread, and there was no evidence that vitamin K was of therapeutic value.

The fourth dog (No. 1-8) received, in addition to vitamin K, a single 200 mg dose of dicumarol 24 hours after irradiation. The effect of this drug was of interest in that a recovery of prothrombic activity did take place before the animal died. It will also be noted that while the prothrombin time recovered in this animal, the whole blood clotting time remained prolonged.

Both toluidine blue and protamine sulfate proved ineffective in overcoming the prolonged prothrombin time induced by dicumarol administration to normal dogs. Six animals were given dicumarol for 2 days and when the prothrombin time was prolonged, toluidine blue was administered to four dogs and protamine sulfate was given to the remaining two animals (Table V). Five of the six dogs died on the 5th and 6th days from massive hemorrhage even though 3 to 5 mg. per kilo of body weight of these substances were administered daily for several days. The failure of these agents to influence the prothrombic activity under these conditions was not surprising. A fact of interest was that the prothrombin reduction in dogs 2-7 and 2-1, was not associated with a marked increase of the whole blood clotting time until after a reduction to less than 20 per cent of normal had occurred. The extensive hemorrhages observed at postmortem seemed out of proportion to the prolongation in the whole blood clotting time, an observtaion emphasizing the fact that the prothrombin time and whole blood clotting times are different expressions of hemorrhagic states and may on occasion be indices to different and unrelated phenomena.

2. Serum Calcium: Although there is no known spontaneous hemorrhagic state attributed to a deficiency of the calcium ion or known to be corrected by the administration of calcium salts, a study of the calcium, phosphorus, and magnesium ions in the irradiated animal was undertaken as a means of exploring all the theoretical possibilities of prolonging the whole blood clotting times. The results of these studies are reported in Table VI. Daily studies

TABLE IV

Failure of Vitamin K to Prevent Hemorrhages in Irradiated Dogs

Dog No. and weight		No. of days after radiation												
		0	1	2	3	4	5	6	7	8	9	10	11	12
8-60	Whole blood clotting time, *min.*....	22	11	1—	10	12	23	10	11	8	—	16	53	
	Prothrombin time, *per cent*.....	100	100	—	100	100	100	100	100	100	—	100	74	
19.0 kg.	Platelet count, *per c.mm*.....	260,000	290,000	—	190,000	130,000	130,000	88,000	96,000	130,000	—	50,000	48,000	
	Vitamin K given, *mg*.	—	5	—	5	5	10*	10*	10*	10*	—	10*	10*	
	Toluidine blue given, *mg*.	—	—	—	—	—	—	—	—	—	—	—	Bleeding	Dead
2-6	Whole blood clotting time, *min*....	18	14	—	10	24	14	12	9	21	—	18	66	Bleeding
	Prothrombin time, *per cent*.....	100	100	—	100	100	100	100	100	100	—	100	100	stopped after dye
17.0 kg.	Platelet count, *per c.mm*	180,000	220,000	—	230,000	140,000	120,000	112,000	120,000	80,000	—	80,000	50,000	injection
	Vitamin K given, *mg*.	—	5	—	5	5	10*	10*	10*	10*	—	10*	10*	
	Toluidine blue given, *mg*.	—	—	—	—	—	—	—	—	—	—	—	60	Dead
2-2	Whole blood clotting time, *min*....	9	8	21	16	30	11	18	—	8	24	47	85	
	Prothrombin time, *per cent*	100	100	84	—	100	100	100	—	100	100	85	95	
7.5 kg.	Platelet count, *per c mm*.	220,000	190,000	220,000	220,000	120,000	120,000	105,000	—	104,000	64,000	64,000	50,000	
	Vitamin K given, *mg*.	—	5	5	10*	10*	10*	10*	—	10*	10*	—	10*	
	Toluidine blue given, *mg*.	—	—	—	—	—	—	—	—	—	—	—	Bleeding	Dead
1-8	Whole blood clotting time, *min*.	24	18	28	35	61	30	71	—	70	65	165		
	Prothrombin time, *per cent*	100	100	42	26	30	7	13	—	10	42	77		
7.5 kg.	Platelet count, *per c.mm*.	340,000	220,000	280,000	340,000	136,000	120,000	120,000	—	64,000	80,000	48,000		
	Vitamin K given, *per c.mm*...	—	5	5	10*	10*	10*	10*	—	10*	10*	—		
	Toluidine blue given, *mg*.	—	—	—	—	—	—	—	—	—	—	—		
	Dicumarol given, *mg*.	—	200	—	—	—	—	—	—	—	—	Bleed-ing		Dead
										(Given 150 cc. of blood)				

* Intravenous administration.

TABLE V

Failure of Toluidine Blue or Protamine Sulfate to Correct Hemorrhage Consequent on Administration of Dicumarol

Dog No. and weight		Control before therapy	No. of days 1	2	3	4	5	6	7	8	9	10	
2-7 21.0 kg.	Prothrombin time, *per cent*	88	—	25	12	13	*Remarks:* Died on 5th day. Developed distemper on 2nd day and massive hematoma formed in right thigh on 3rd day.						
	Whole blood clotting time, *min.*	20	—	19	23	22							
	Dicumarol given, *mg.*	300	—	200	—	—	42						
	Platelet count *per c.mm.*	190,000	—	180,000	170,000	135,000							
	Toluidine blue given, *mg.*	—	—	—	50	50							
2-1 8.0 kg.	Prothrombin time, *per cent*	100	—	28	5	12	16	*Remarks:* Dog died on 6th day from massive hemorrhage.					
	Whole blood clotting time, *min.*	16	—	22	90	45	55						
	Platelet count, *per c.mm.*	260,000	—	270,000	190,000	340,000	460,000						
	Dicumarol given, *mg.*	200	—	200	—	—	—						
	Toluidine blue given, *mg.*	—	—	—	50	50	—						
1-9 6.9 kg.	Prothrombin time, *per cent*	100	—	48	15	5	*Remarks:* Developed a large hematoma on 3rd day in right thigh. Hematocrit reading on 3rd day was 54 and it fell to 41 on the 4th day. Died on 5th day.						
	Whole blood clotting time, *min.*	28	—	25	36	85							
	Platelet count, *per c.mm.*	240,000	—	240,000	200,000	190,000							
	Dicumarol given, *mg.*	200	—	150	—	—							
	Toluidine blue given, *mg.*	—	—	—	50	50							
1-7 9.0 kg.	Prothrombin time, *per cent*	100	—	40	27	20	60	83	100		100	100	
	Whole blood clotting time, *min.*	17	—	—	50	45	35	15	21		16	24	
	Platelet count, *per c.mm.*	290,000	—	250,000	300,000	400,000	480,000	490,000	460,000		—	—	
	Dicumarol given, *mg.*	200	—	200	—	—	—	—	—		—	—	
	Toluidine blue given, *mg.*	—	—	—	50	50					—	—	*Remarks: Dog survived.*
6-3 10.6 kg.	Prothrombin time, *per cent*	100	—	28	10	8	*Remarks:* Developed large retroperitoneal hematoma and died on 5th day.						
	Whole blood clotting time, *min.*	21	—	26	31	29							
	Dicumarol given, *mg.*	200	—	200	—	—							
	Platelet count, *per c.mm.*	290,000	—	260,000	270,000	290,000							
	Protamine sulfate, *mg.*	—	—	—	50	50							
6-4 9.7 kg.	Prothrombin time, *per cent*	100	—	34	13	10	6	*Remarks:* Died on 6th day from massive hemorrhage.					
	Whole blood clotting time, *min.*	18	—	19	26	30	35						
	Dicumarol given, *mg.*	200	—	200	—	—	—						
	Platelet count, *per c.mm.*	260,000	—	270,000	240,000	270,000	260,000						
	Protamine sulfate, *mg.*	—	—	50	50	50							

were not carried out on all animals, although in no case was there more than a 3 day period between determinations. The figures presented are averages based on the individual results obtained on all animals tested on a given day. The serum calcium was determined by the method of Cramer and Tisdell (12), the serum phosphorus by the Bodansky procedure (13), and the serum magnesium by Hoffman's method (14). No significant deviations from the normal values were observed, although the tendency to hemorrhage progressed until

TABLE VI

Average Values of Serum Calcium, Phosphorus, and Magnesium in Irradiated Dogs

Day	No. of dogs tested	Serum			Clotting time	Platelet count	Final outcome
		Calcium	Phosphorus	Magnesium			
		mg. per cent	*mg. per cent*	*mg. per cent*	*min.*	*per c. mm.*	
Control	13*	11.4	3.7	2.0	17	340,000	
1st	5	10.4	3.8	2.8	10	299,000	
2nd	6	11.1	3.6	2.2	14	207,000	
3rd	10	10.8	4.1	2.2	29	197,000	
4th	8	10.2	3.9	2.2	19	149,000	
5th	6	10.5	3.5	2.1	21	130,000	
6th	7	10.2	4.3	2.2	19	114,000	One dog died
7th	9	9.7	3.5	2.2	33	133,000	One " "
8th	8	10.8	3.8	2.2	24	99,000	Three dogs "
9th	5	10.3	3.4	2.2	48	84,000	Two " "
10th	5	10.2	3.8	1.9	47	76,000	One dog "
11th	4	10.2	2.8	2.2	55	59,000	
12th	4	10.0	4.0	2.6	53	52,000	" " "
13th	2	10.5	3.7	2.6	35	44,000	Two dogs "
16th	2	10.7	4.5	2.1	31	45,000	Both dogs partially treated for hemorrhage

*Two control determinations were made on each animal during the week immediately preceding irradiation.

death. Four exceptions were noted in four dogs not included in Table VI. The serum calcium levels in these animals were reduced, but these changes occurred before hemorrhage developed. Four of the animals reported in Table VII were given calcium gluconate when hemorrhage appeared, but no improvement was noted.

3. Plasma Fibrinogen: The fibrinogen level was not determned. The fact that an ample clot formed indicated the presence of fibrinogen. Clot retraction, however, was retarded or completely absent and this defect was closely related to the platelet count.

There was some evidence that the clot itself was not entirely normal in the blood of the untreated irradiated dog. The blood appeared to gel before an

actual clot was formed. The gelled blood could be reverted to a fluid state if the blood was shaken before a solid clot had formed. Considerable time often elapsed after the gel appeared before firm clotting took place. Shreds of fibrin did not appear until shortly before the solid clot appeared. This phenomenon, however, was not studied extensively, though the observation was made that it occurred also in slightly heparinized normal dog blood in which coagulation was delayed. It was not observed in prothrombin-deficient dog blood or in human hemophilic blood in which the whole blood clotting time was prolonged. Once the clot began to form in these latter two conditions it was rapidly completed without visibly exhibiting any tendency to first gel.

4. Platelets: The total number of circulating platelets is reduced after marked exposure to x-irradiation, and aplasia or the marrow may result (5, 6). While the bleeding irradiated dogs of our series always developed a profound thrombocytopenia, this reduction of platelets did not always coincide with the onset of bleeding. In some animals bleeding preceded thrombocytopenia, in others it developed afterwards, but the majority showed the reduction of platelets at about the time hemorrhage appeared. This relation was not that of cause and effect, as was demonstrated in dog 1-08. In this animal the platelet count was 260,000 when hemorrhage began and the blood became incoagulable.

Once hemorrhage developed and the clotting time was prolonged, freshly drawn transfusions of citrated whole blood had little effect. Of all the measures employed, only toluidine blue or protamine sulfate proved effective. Certain other members of the thionin series (azure A, azure B, thionin, and to some extent methylene blue) were also tested and showed some antihemorrhagic effect, but, save for methylene blue, they proved too toxic for therapeutic use.

The lack of a cause and effect relation between the thrombocytopenia and the hemorrhagic state was also brought out by the fact that toluidine blue or protamine sulfate stopped the tendency to bleed but did not elevate the platelet count. In Table VII are presented data on nine dogs whose clotting times returned to normal after dye injecton without any rise in the platelet count. This same phenomenon was even better demonstrated in animals given repeated injections of either toluidine blue or protamine sulfate which controlled the hemorrhage even though the thrombocytopenia was marked. For example in dog 5-1 the clotting time was returned to normal on five occasions although the platelet count remained severely reduced from the seventh postirradiation day until death (Text-fig. 2).

It would appear that the thrombocytopenia associated with x-irradiation, in the dog at least, did not materially contribute to the prolonged clotting time. Just what may be the significance of the thrombocytopenia with reference to the hemorrhagic state in this condition remains to be seen. The thrombo-

TABLE VII

Effect of a Single Injection of Toluidine Blue on the Whole Blood Clotting Time in Irradiated Dogs with Hemorrhage

Dog. No.	Weight	Total amount of dye given	Whole blood clotting times			
			Before dye injection	24 hrs. after injection	48 hrs. after injection	72 hrs. after injection
	kg.	*mg.*				
1-03	11.2	10				
Whole blood clotting time, *min.*			84	11	90	—
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			20,000	15,000	15,000	
1-08	7.4	24				
Whole blood clotting time, *min.*			Incoagulable	4		
Prothrombin time, *per cent*			100	100		
Platelet count, *per c.mm.*			70,000	70,000		
3	11.0	50				
Whole blood clotting time, *min.*			40	26	15	
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			110,000	96,000	72,000	
9-45	11.2	50				
Whole blood clotting time, *min.*			56	20	36	
Prothrombin time, *per cent*			100	100	100	
Platelet count, *per c.mm.*			48,000	32,000	40,000	
1-0	10.6	75				
Whole blood clotting time, *min.*			35	4		
Prothrombin time, *per cent*			100	100		
Platelet count, *per c.mm.*			90,000	70,000		
5-1	15.0	80				
Whole blood clotting time, *min.*			51	2	1	14
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*						
7-4	15.5	80				
Whole blood clotting time, *min.*			120	4	6	32
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*			90,000	60,000	60,000	—
6-5	9.5	50				
Whole blood clotting time, *min.*			25	16	11	32
Prothrombin time, *per cent*			100	100	100	100
Platelet count, *per c.mm.*			200,000	200,000	200,000	86,000

TABLE VII—*Concluded*

Dog. No.	Weight	Total amount of dye given	Whole blood clotting times			
			Before dye injection	24 hrs. after injection	48 hrs. after injection	72 hrs. after injection
	kg.	*mg.*				
4-99		100				
Whole blood clotting time, *min.*			28	9	29	
Prothrombin time, *per cent.*			100	100	100	
Platelet count, *per c.mm.*			160,000	150,000	140,000	
5		40				
Whole blood clotting time, *min.*			64	150	170	
Prothrombin time, *per cent.*			8	5	2	
Platelet count, *per c.mm.*			96,000	100,000	80,000	

TABLE VIII

Results of the Attempted Isolation of Heparin from the Blood of Irradiated Dogs with Hemorrhage

Dog. No.	Volume blood	Whole blood clotting time on day of death	Heparin-like material obtained	Units per mg.
		min.	*mg.*	
9-5	475	87	11	110
9-6	860	65	9	100
9-7	320	68	6	110
9-8	430	47	4	90

cytopenia may well have been responsible for the impaired clot retraction and the prolonged bleeding time, neither of which responded to protamine sulfate or toluidine blue administration.

(c) *Attempts to Isolate Heparin.*—Reported below is our experience with attempts at heparin isolation from the blood of irradiated dogs with hemorrhage. The procedure of Jaques and Waters (15) was employed for this purpose. Efforts at isolating heparin from blood were complicated by the fact that in most animals the blood was not rendered entirely incoagulable but that instead the clotting time was prolonged to three to five times normal. This fact, coupled with the relatively inefficient method of heparin recovery, made this part of our problem especially difficult.

Four dogs which had received 450 r irradiation over the entire body were exsanguinated under local anesthesia with novocaine, and the bloods obtained were separately analyzed for the amorphous sodium acid salt of heparin. The resulting data are presented in Table VIII. The anticoagulant property of the amorphous material obtained in each case was high, and when toluidine blue or

protamine sulfate was added to it in solution, its anticoagulant property was lost. About 3 mg. of dye was necessary to inactivate 2 mg. of the anticoagulant. This same ratio held also for protamine sulfate. In the case of the dye a small metachromatic precipitate formed in the bottom of the test tube about 20 minutes after the dye had been added. The anticoagulant was heat-stable at 100°C. for 20 minutes.

The inefficiency of the method of isolation in our hands was demonstrated by the fact that in nine attempts at recovery of heparin from normal blood to which a standard sodium acid salt of heparin had been added, only 5 to 15 per cent of the original material was recaptured. The greatest loss appeared to take place in the initial procedure of protein precipitation.

DISCUSSION

The experimental observations here reported suggest that the defect responsible for the hemorrhagic state in the irradiated dogs was the presence of an anticoagulant in the blood. This substance so far as it was tested, resembled heparin.

Heparin has been obtained in high degrees of purity by many workers, yet it is still known mostly by its biologic properties. It is thought to be a polysaccharide with many sulfuric acid groups, and is classified as an ester of mucotin sulfuric acid, but its chemical identity is not yet known (11). Certain of its constituents, especially its sulfur content and its crystalline characteristics are at the moment under controversy. While the material isolated from the blood of our animals seemed in no way dissimilar from a standard sodium heparin salt, we can class it only as "heparin-like" because the identity of heparin itself is unknown.

Different lots of toluidine blue have varied considerably in their antiheparin activity *in vitro*. Many samples of toluidine blue showed no activity even though they were obtained from the same source. The intravenous administration of the dye, however, controlled bleeding even though the antiheparin activity was slight on *in vitro* titration, but a longer period of time was required to control bleeding in the case of the less active preparations. Titrations of heparin activity with protamine sulfate, however, were always satisfactory, and while some variations occurred from lot to lot, each new lot could readily be standardized against the standard heparin preparation.

It was not the purpose of these experiments to increase the survival periods of fatally irradiated dogs, although the data suggest that when hemorrhage was controlled life may have been prolonged; the administration of toluidine blue or protamine sulfate at fairly frequent intervals lengthened life by an average of 10 days. The dose of 450 r x-ray used was approximately 150 to 175 r greater than the LD_{50} for dogs (300 r) and resulted in the death of the untreated animal after 11 days on the average. At 450 r there were no survivals in either the

control group or the animals given toluidine blue or protamne sulfate. The cause of death in these animals was not determined. Infection in the terminal phase was always a prominent finding, but probably other factors were involved. It is known that in less severely injured animals (dogs and rabbits) the marrow will recover to a large extent, even though at the height of the disturbance the histologic evidence of injury may be just as great in the animal which survives as in that which dies (7). This being so, the control of hemorrhage in the less severely irradiated animals may contribute to the animal's recovery.

SUMMARY

When the entire body of dogs was exposed to 450 units of Roentgen irradiation a hemorrhagic syndrome developed which was characterized by thrombocytopenia, prolonged clotting and bleeding times, and neutropenia.

The prothrombin time remained normal until about 24 hours before death. The calcium, phosphorus, and magnesium levels were not altered. Fibrinogen was present but syneresis was poor.

Toluidine blue and protamine sulfate, substances which can inhibit the biologic action of heparin, restored the clotting time to normal.

The hemorrhagic state was not materially altered by transfusions, vitamin K, or vitamin C.

Toluidine blue and protamine sulfate were ineffective in the control of hemorrhage produced by dicumarol.

The defect responsible for bleeding after irradiation appeared to be the presence in the circulation of an anticoagulant whose properties, so far as tested, were indistinguishable from those of heparin.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Typical picture of gastrointestinal tract of untreated irradiated dog (450 r).

(Allen *et al.*: Heparinemia (?))

PLATE 2

FIG. 2. Gastrointestinal tract of irradiated dog (450 r) treated with frequent injections of toluidine blue. Note, however, that the edema and thickening of the gut wall persist.

(Allen *et al.*: Heparinemia (?))

STUDIES
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THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Announcement

Volumes 131 and 132 of the *Studies from The Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

A STUDY OF NERVE PHYSIOLOGY

The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1000 pages with 480 illustrations.

In order that the volumes may be available to those who do not receive them as part of their subscriptions to the *Studies*, extra copies have been printed which are purchasable at the regular price of $2.00 per volume. The two volumes will only be sold together. They can be obtained from the Publication Service, The Rockefeller Institute for Medical Research, York Avenue and 66th Street, New York 21, N. Y., at a price of $4.00, payable in advance.

THE LETHAL EFFECT OF RELATIVE HUMIDITY ON AIR-BORNE BACTERIA*

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An understanding of the influence of atmospheric conditions on the viability of air-borne microorganisms is essential to any attempt to formulate the epidemiology of air-borne infections. The influence of the relative humidity of the atmosphere has received inadequate quantitative study and conflicting effects have been attributed to it in promoting survival or destruction of air-borne pathogenic agents. Thus Williamson and Gotaas (1) concluded from their observations on *Serratia marcescens*, *Escherichia coli*, *Staphylococcus albus*, *Staphylococcus aureus*, and *Streptococcus salivarius*, that low relative humidities are more favorable than high ones for the viability of air-suspended microorganisms. Similarly, Edwards *et al.*, and Loosli and his associates (2) reported that influenza virus dispersed into the air is killed much more swiftly in humid than in dry air, and DeOme (3) found that the death rate of *Salmonella pullorum* atomized from aqueous suspension increases steadily with increase in the relative humidity from 15 to 80 per cent. On the other hand, Wells and Zappasodi (4) stated that hemolytic streptococci sprayed into the air are rapidly killed in dry atmospheres but are protected in the presence of moisture. In few of these investigations were many points on the relative humidity scale examined and in some cases, the exact values of the atmospheric moisture content were not determined. The marked influence of water vapor on the action of aerial germicides (5, 6) also makes desirable more careful study of its effects on microorganisms.

In the present study bacterial suspensions were atomized into an experimental chamber under controlled conditions of temperature and relative humidity, and the concentration of viable microorganisms remaining in the air after various time intervals was measured.

Methods

The experiments were carried out in the 640 cubic foot chambers previously described (7) which allow precise control of temperature and relative humidity over fairly wide ranges.

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Seven and a half hour cultures of microorganisms were grown in heart infusion broth (Difco) containing added serum and dextrose, and suspensions prepared from these cultures were atomized into the experimental chambers for 1 minute by the standard technique already described (7). The bacterial suspensions were dispersed by reflux type atomizers (8) whose particle size distribution was measured by means of a cascade impactor[1] (9).

By careful standardization of the spraying procedure it was found possible to introduce a fairly constant number of microorganisms into the 640 cubic foot chamber in each experiment. This number was fixed at about 5 million. Daily checks on the total number of microorganisms discharged by the atomizer were carried out by repeating the spraying procedure with the atomizer connected directly to a bubbler sampler (10), so that the entire spray was absorbed in the bubbler fluid, aliquots of which were then plated in nutrient agar. The bacterial content of the air was determined over a 2 hour period after atomization of the microorganisms, by means of successive 2 minute bubbler samples and by exposure of agar settling plates (7).

TABLE I

Survival of Pneumococci, Type I, Sprayed from Broth Suspension into Air at a Temperature of 22.2°C. and a Relative Humidity of 19 Per Cent

Mass median diameter of the bacterial spray emergent from the atomizer was 3.2 μ.

Time of sample (interval between end of spray and start of sample)	No. of pneumococci per cubic ft. of air recovered by bubbler sampler	No. of pneumococci collected on a 5 min. settling plate
min.		
0	5200	332
2	4760	
5	4580	290
10	4000	247
20	3800	193
30	3240	177
45	2700	152
60	2420	106
75	2240	96
90	1940	90
105	1800	74
120	1620	66

The survival curves obtained by these two sampling methods paralleled each other very closely. By and large, the bubbler sampler values were somewhat more uniform, and so these have been used for calculations of the survival constants which will be reported here. A typical experimental protocol is shown in Table I.

The relative humidity was varied over a range extending from 3 to 80 per cent at three different temperatures, 14.4, 22.2 and 33.3°C. For those regions of temperature and humidity where very high bacterial death rates were encountered, the results were checked by many repetitions of each experiment.

EXPERIMENTAL RESULTS

The most striking fact revealed by these tests was the demonstration of the existence of a narrow range of relative humidity in the vicinity of 50 per cent

[1] The same atomizer was employed for all the experiments here described, except for those in which the effect of changing particle size was studied.

which is rapidly lethal for microorganisms freshly sprayed into the atmosphere from a broth suspension. The pneumococcus, a relatively delicate microorganism, exhibited the greatest sensitivity to this killing action of the atmosphere, while that of the streptococcus group C and staphylococcus was much less pronounced. In Fig. 1 are presented typical results of experiments with the pneumococcus at three different relative humidities, which illustrate the capacity of the microorganism to survive for long periods at both very low and very high relative humidities, but not at intermediate values.

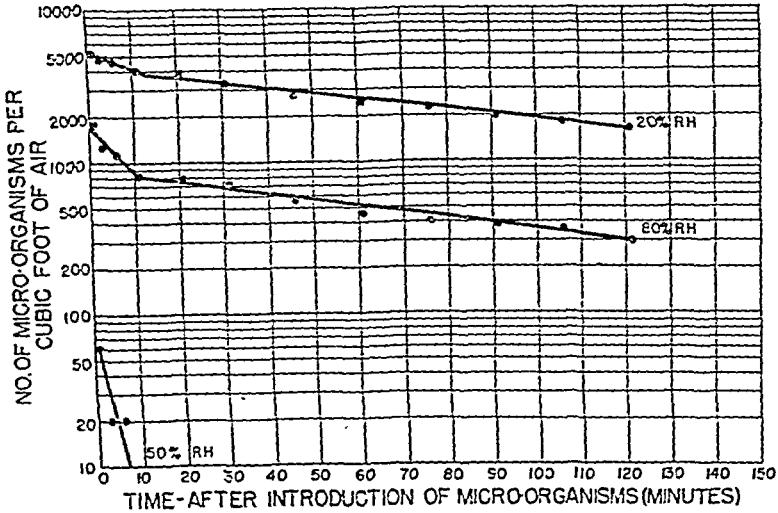

FIG. 1. Logarithmic plot of the survival of pneumococci sprayed from broth culture into atmospheres of various relative humidities, at 22.2°C. The cloud leaving atomizer had a mass median diameter of 3.2 μ. Approximately equal numbers of microorganisms were introduced into the chamber in each experiment.

Analysis of these survival curves reveals that at least two separate rate-determining lethal processes are involved. The initial one is always more rapid, and produces fairly extensive killing throughout the first 5 to 20 minutes after the droplets containing the microorganisms have been introduced into the air. The second decay process proceeds more slowly and generally lasts throughout the 2 hour observation period (Fig. 1). Both killing processes give linear logarithmic survival curves so that when the number of survivors is plotted as a logarithmic function of the time, the resulting curve consists of two straight lines meeting at a point somewhere between 5 and 20 minutes after the introduction of the microorganisms into the chamber. The slopes of these two parts of the curves have been found to be influenced by the relative humidity and temperature of the air, the nature of the microorganisms employed, the composition of the liquid medium in which they are suspended, and the

particle size of the droplets in which they are contained when sprayed into the atmosphere. Experimental effects demonstrating the operation of these factors have been studied in greatest detail for pneumococci.

Experiments similar to those presented in Fig. 1 were carried out at many different relative humidities, using a serum-dextrose broth suspension of a

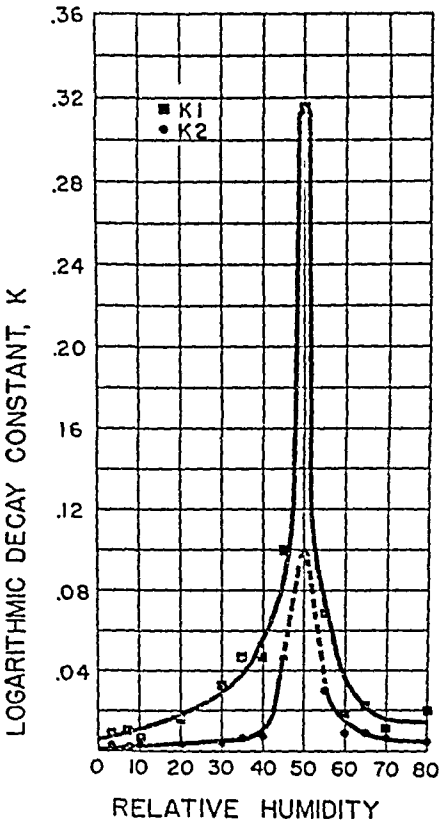

FIG. 2. Slopes of the logarithmic survival curves for pneumococci sprayed from broth suspension into atmospheres of various relative humidities. K_1 is the slope of initial part of the survival curve, and K_2 is the logarithmic decay constant for the final portion of the curve. The value shown for K_1 at 50 per cent relative humidity, 0.32 ± 0.12, is the averaged result of more than a dozen experiments. Accurate values for K_2 in the central humidity range could not be determined, because so few microorganisms survived beyond 20 minutes.

$7\frac{1}{2}$ hour pneumococcus culture. For each experiment, the numbers of survivors were plotted as a logarithmic function of the time (as shown in Fig. 1) and values for the two constants, K_1 and K_2 (which respectively represent the logarithmic rates of disappearance of viable microorganisms from the air during the first 10 to 15 minutes (K_1) and for the interval thereafter (K_2)) were determined at each relative humidity at 22.2°C. The atomizer employed in these tests produces a cloud of bacteria suspended in broth droplets, whose mass

median diameter[2] is 3.2 μ, on issuing from the atomizer. A plot of the slopes of these survival curves is presented in Fig. 2. It shows that within the narrow range of relative humidities between 40 and 55 per cent, the death rate of these microorganisms sprayed in the air from a broth suspension is enormously accelerated.

Effect of the Type of Microorganism.—Staphylococcus albus and *Streptococcus hemolyticus* group C exhibited the same kind of logarithmic survival curves as that shown for the pneumococcus in Fig. 1. The effect of the relative

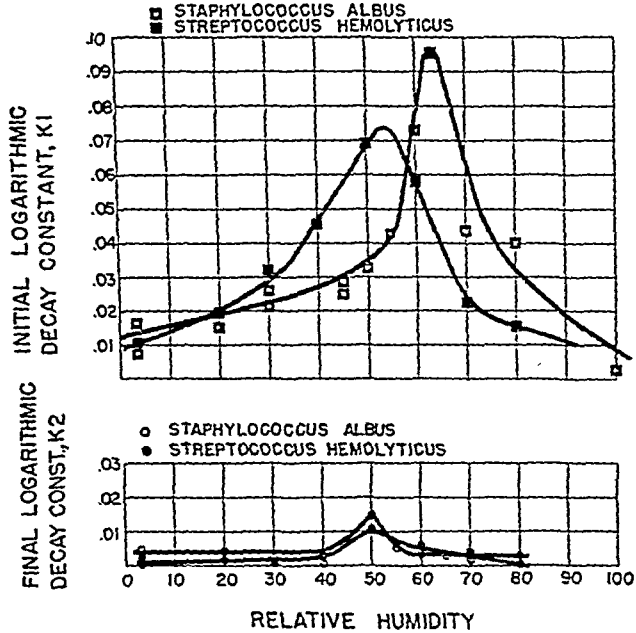

FIG. 3. Logarithmic decay constants, K_1 and K_2 of hemolytic streptococcus group C, and *Staphylococcus albus*, as a function of the relative humidity of the atmosphere. Microorganisms were sprayed from broth suspension.

humidity on the death rates of these microorganisms also followed the same pattern. The only observed difference lay in the relative magnitudes of the effect and in slight displacement of the value of the relative humidity producing the maximum death rate. The order of increasing susceptibilty to this killing action of intermediate relative humidity was: hemolytic streptococcus group C, staphylococcus, and pneumococcus Type I. The effect of various relative humidities on the survival constants of the streptococcus and staphylococcus is presented in Fig. 3.

[2] *I.e.* 50 per cent of the mass of the cloud was contained in droplets whose diameter was less than 3.2 μ.

Effect of Settling.—It is necessary to know to what extent this disappearance of the microorganisms from the air represents a real lethal process, rather than simply a removal of air-suspended particles by settling or coalescence. In order to test this point the series of experiments represented by the points of Fig. 2 was repeated with a broth culture of microorganisms to which methylene blue had been added. Samples of the chamber air were bubbled through a solution of 70 per cent alcohol in H_2O, at the same time intervals as those used in the previous experiments. Then the total amount of dye collected in each of these samples was determined photometrically In this way the rate of removal of these particles from the air by inelastic collisions with the walls and

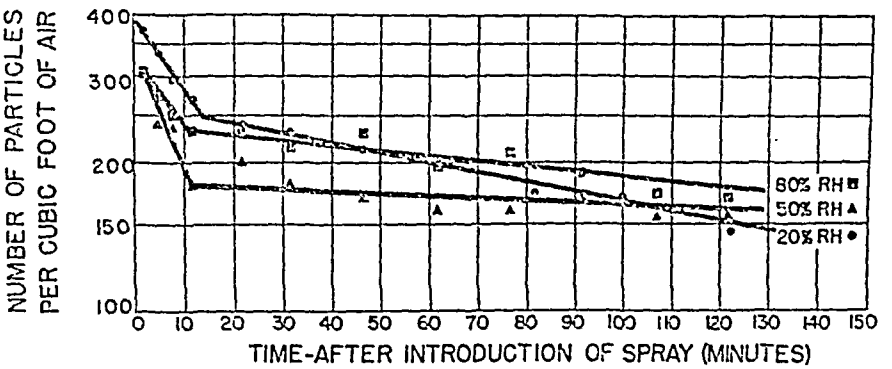

FIG. 4. Determination of settling rates by measurement of the persistence in the air of sprayed droplets containing microorganisms and a dye, at various relative humidities. The suspending medium was broth, and the instantaneous mass median diameter of the cloud was 3.2 μ. (The ordinate units are arbitrary, the figures simply indicating the relative amounts of dye present in a cubic foot of air, as a function of the time.)

floor of the chamber was determined. Sample plots of these data are shown in Fig. 4. The logarithmic curves so obtained also consist of two straight lines with the break occurring at a point usually between 10 and 20 minutes. However, when as in Fig. 5, the slopes of these curves are plotted for the entire range of relative humidity, no maximum occurs in either K_1 or K_2 as was found for the recovery of viable microorganisms from the air. The rate of settling remains low at all relative humidities,[3] whereas the rate of disappearance of viable pneumococci, for example, may be increased by 50- to a 100-fold at intermediate relative humidities. Since the rapid disappearance of microorganisms sprayed from a broth suspension into atmospheres of intermediate relative humidity cannot be accounted for by settling or collision processes, a true lethal

[3] It is especially low below 12 per cent relative humidity. The sharp break in the curve at this point would indicate that complete dehumidification of these particles occurs at this relative humidity.

action must be operating at relative humidities in the neighborhood of 50 per cent.[4]

An explanation for this rapid killing of air-borne microorganisms which occurs in atmospheres of intermediate relative humidities must be sought in an analysis of the processes which occur when bacteria-laden droplets are introduced into unsaturated air. Evaporation of water from such droplets first results in an increase in the concentration of any soluble substances present in the fluid surrounding the microorganism. If sufficient water is lost, one or more of these solutes may attain a concentration which is toxic to the cell. However, at the same time, water is also lost from the bacterial cell itself. If the cell becomes highly desiccated it becomes resistant to many kinds of physical

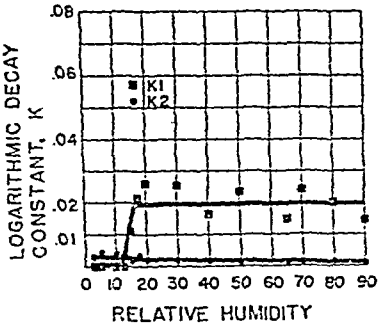

FIG. 5

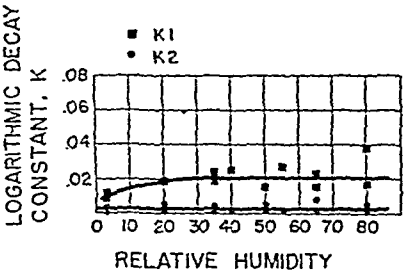

FIG. 6

FIG. 5. Slopes of the settling curves of droplets of a 3.2 μ cloud introduced into atmospheres of varying relative humidities. K_1 is the slope of the initial part of the curve, and K_2 the logarithmic decay constant for the final period. The difference between this curve and that of Fig. 2 indicates that the increased rate of disappearance of viable microorganisms from the air at intermediate relative humidities cannot be due to a settling process.

FIG. 6. Logarithmic survival constants of pneumococci sprayed from a distilled H_2O suspension as a function of the atmospheric relative humidity.

and chemical stresses (11) and so acquires a degree of immunity to the destructive action of the high solute concentration.

The amount of water lost by such a cell depends upon the atmospheric

[4] These data on settling also suggest that the more rapid rate of disappearance of a bacterial suspension during the first 10 to 20 minutes after its atomization (i.e. *the difference* between K_1 and K_2) is due to processes which selectively affect the larger particles more than the smaller ones. Settling of the larger particles of the cloud during the first 10 to 15 minutes undoubtedly accounts for the increased initial rate of disappearance of the dye aerosol. The same processes plus any influence which the larger particle size *per se* may exert in hastening the killing of bacteria contained within the droplets would account for the existence of the two separate death rates, K_1 and K_2, which are characteristically observed when a cloud of bacteria-containing droplets is introduced into the atmosphere under the conditions described here (see below, section on particle size).

relative humidity and the binding energy between water and the various cellular constituents. At very low relative humidities dehydration is so extensive that a stable state may be achieved wherein the microorganism is not affected by the high concentration of solutes which remains. Conversely, at high relative humidities, enough water is bound to and about the cell so that lethal conditions are not achieved. The experiments here described suggest that at intermediate humidities, the amount of water retained in intimate association with the microorganism produces a critical state in the cell. In this condition the bacterium becomes highly susceptible to the toxic action of the unphysiologic concentration of solutes remaining from the original medium in which it was suspended.

Effect of Varying the Composition of the Suspending Fluid.—In the light of the foregoing analysis, it may be predicted that if the microorganisms are sprayed into air from a suspension in distilled water rather than broth, the rise in death rate at the intermediate relative humidities should be greatly reduced. This conclusion was verified experimentally. Seven and one-half hour cultures of pneumococcus, Type I, were centrifuged, washed once in distilled H_2O, and then resuspended in water before spraying into the experimental chamber. The survival rate was then determined in the standard manner. The characteristic linear logarithmic survival curves exhibiting a sharp break at the end of 10 or 15 minutes were obtained, exactly like those observed in the broth experiments. However, in contrast to these latter tests, substitution of distilled water for broth completely eliminated the great increase in death rate at humidities around 50 per cent. The variation with relative humidity of the logarithmic survival constants of pneumococci sprayed into the air from a distilled water suspension is shown in Fig. 6. Comparison of the curves of Figs. 6 and 5 reveals that the survival pattern of these air-borne pneumococci is practically identical with that obtained for the persistence of an aerosol of dye droplets, indicating the absence of any lethal process.

It became of interest to ascertain which of the components of the broth solution was exerting the toxic effects observed at relative humidities in the vicinity of 50 per cent. Separate solutions of the various constituents of the nutrient broth were prepared and a solution of each component in turn, in the same concentration in which it occurs in the broth, was employed as a suspending medium from which pneumococci were sprayed into the air. Such experiments revealed that NaCl present in the original solution in a concentration of 0.5 per cent, was the agent largely responsible for the lethal action. All the other constituents of the nutrient broth, when tested either singly or together failed to produce a marked lethal effect at intermediate relative humidities. Thus determination of the survival constants of a pneumococcus culture sprayed into the air from a suspension containing all the components of

nutrient broth except NaCl,[5] resulted in a pair of curves like that of Fig. 6. Conversely, when a solution containing only 0.5 per cent NaCl was employed survival curves superposable on those of Fig. 2 resulted. Thus the characteristic sharp rise in mortality rate at intermediate relative humidities occurs only in the presence of the NaCl.

In the light of these results, it was important to test the effect of relative humidity on bacteria sprayed into the air from a saliva suspension. This fluid constitues the natural medium from which respiratory bacteria are introduced into the atmosphere, so that the survival pattern of microorganisms suspended in it would have direct bearing on problems of natural air-borne infection. Saliva was collected from human volunteers who chewed paraffin for several minutes to induce a rapid flow. No attempt was made to free the fluid from its normal bacterial population, since in the final suspension which was prepared, these microorganisms were so far outnumbered by the pneumococci as to represent a negligibly small degree of contamination. A 7½ hour culture of pneumococci Type I was spun down and resuspended in an equal volume of saliva. The resulting suspension was immediately transferred to the atomizer and sprayed into the chamber by the standard procedure used in the preceding experiments. The resulting survival pattern was identical with that obtained when broth or saline (Fig. 2) was used as the suspending medium, rather than the distilled water type of curve (Fig. 6).

Effect of the Size of the Droplet.—If death of bacteria sprayed into the air from a broth or saliva suspension is indeed due to a process connected with dehydration then an atmospheric relative humidity which is ordinarily lethal should be much less so when the bacteria are suspended in small droplets rather than in large ones. This follows because the smaller droplet, containing a smaller total amount of the injurious agents exposes the microorganism to a lower concentration of them as dehydration proceeds. This effect was tested by comparing the survival rates of pneumococci sprayed into the air from a serum-dextrose broth suspension, by two different atomizers, delivering clouds whose instantaneous particle size distributions corresponded to mass median diameters of 1.6 μ and 3.2 μ respectively. A typical set of experiments at 22.2°C. and 50 per cent relative humidity is presented in Fig. 7, demonstrating the greater rate of killing for the pneumococci suspended in the larger droplets. In a series of over fifteen experiments at 22.2°C. the average value of K_1, the decay constant observed during the first 10 to 20 minutes after the end of the spray, was 0.012 $\pm$ 0.005 per minute for the 1.6 μ particles, and 0.32 $\pm$ 0.12 per minute for those 3.2 μ in diameter. The average value of K_2, the killing rate during the second part of the survival curve was 0.045 per minute for the 1.6 μ particles. (So few bacteria remained alive at the end of 20 minutes that

[5] *I.e.* this solution contained 1 per cent peptone, 1 per cent tryptose, 0.05 per cent dextrose, and 2.0 per cent serum.

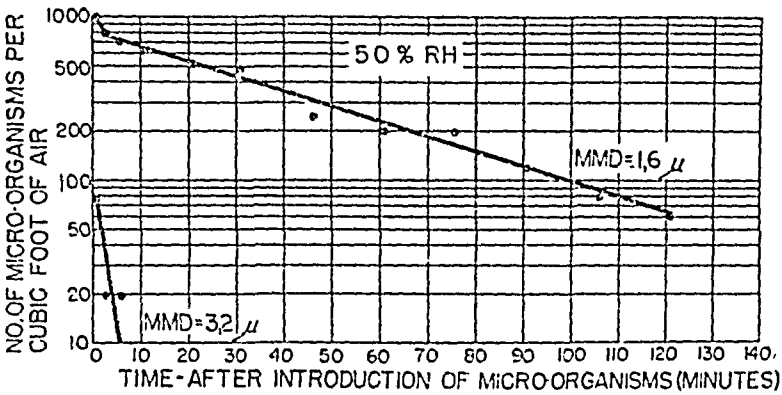

FIG. 7. Effect of cloud particle size on the survival of pneumococci sprayed from broth suspension into an atmosphere of 50 per cent relative humidity at 22.2°C.

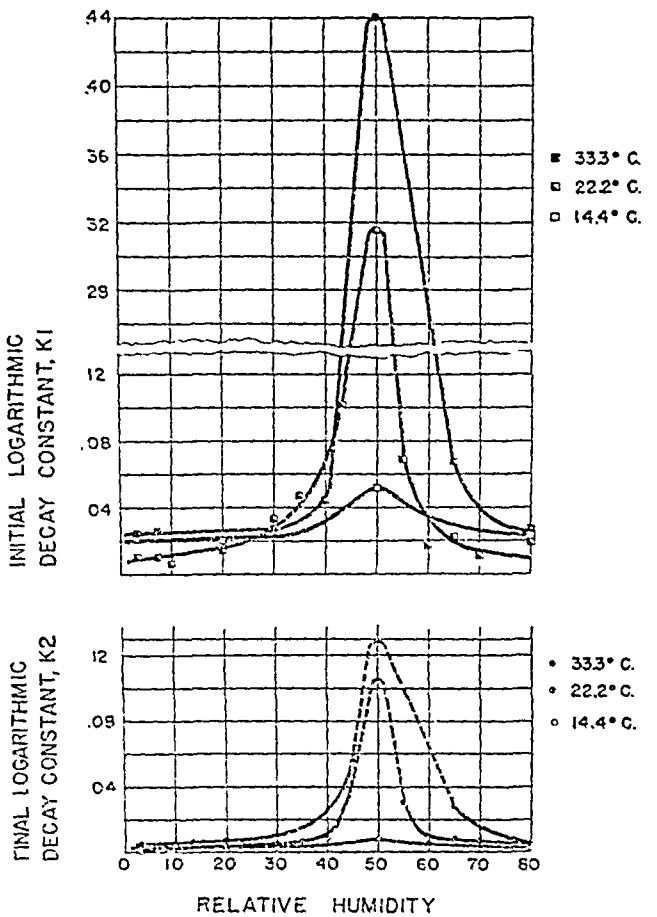

FIG. 8. Effect of temperature on the survival constants of pneumococci sprayed from broth suspension into atmospheres of varying relative humidity.

it was impossible to obtain a satisfactory value of K_2 for the 3.2 μ particles at 50 per cent relative humidity.)

Effect of Temperature.—The influence of temperature changes on this lethal process was also studied. The survival of suspensions of pneumococci in broth sprayed into the air at various relative humidities, was measured at 14.4°C. and 33.3°C., as well as at 22.2°C. As might be expected a pronounced temperature coefficient was observed, K_1 varying by a factor of fifteenfold between these temperature extremes. Values for K_1 and K_2 as a function of the relative humidity at the three temperatures are contained in Fig. 8. As before, the values for K_2 in the intermediate humidity zone are uncertain at the two higher temperatures, since the extremely high initial death rates under these conditions leave too few survivors to enable the subsequent lethal process to be studied.

DISCUSSION

When a suspension of a given composition is atomized into the atmosphere the ultimate extent of dehydration depends on the relative humidity, since the droplet eventually comes into a condition of equilibrium with the atmosphere. When this occurs, the water content of the particle will be such that the aqueous tension within it is equal to the partial pressure of water in the air. At low relative humidities, then, a particle containing a microorganism will have lost almost all its water—even some which may have been in fairly tight chemical combination with certain cellular constituents. At high relative humidities, however, even very loosely bound water will be retained. At intermediate relative humidities, partial dehydration of the various cellular systems will occur. Calculation shows that even at a relative humidity of 50 per cent, spherical droplets of distilled water can evaporate from an initial diameter of 3 μ to one-tenth that size within 0.004 second.[6] This time is much faster than any of the death rates observed in the experiments reported here so that the evaporation process would be finished before the lethal process was well under way. Hence, the rates of killing observed in these experiments must be interpreted as constituting the natural mortality of the microorganisms in a given chemical environment, when in a state of hydration which is in equilibrium with the prevailing relative humidity.

If the theoretical formulation here proposed is correct, then the lethal action

[6] The rate of evaporation of small droplets in unsaturated air has been shown to proceed according to the equation (12): $\frac{dA}{dt} = \frac{8\pi DM}{RTd}(p_s - p'_s)$ where A = area of the droplet, D = diffusion coefficient of the vapor, equal to 0.24 cm² per second for water vapor in air at room temperature; M = molecular weight of the vapor, 18.0 for H_2O; R = gas law constant, 62,400 cc. × mm. Hg. mole^{-1} deg.$^{-1}$; T = absolute temperature, 295° A; d = density of the lique-

observed at intermediate relative humidities is not due simply to the salt activity by itself. Droplets of pure sodium chloride solution sprayed into the air become saturated with this solute at a relative humidity of 75 per cent (13) and at lower humidities would dry out completely. However, in our experiments microorganisms suspended in pure saline showed a maximum lethal effect at relative humidities near 50 per cent rather than at 75 per cent. This apparent discrepancy can be explained if one considers that in a bacterial suspension the salt enters into solution and association with some of the cellular constituents. The maximum lethal action of the salt which is bound within the cell, is exercised at the relative humidity which dehydrates the microorganism to the point where it becomes most vulnerable, rather than at one which is in equilibrium with a saturated solution of pure NaCl.

The present experimental results have been explained by postulating the existence of a critical degree of moisture content at which a bacterial cell becomes much more susceptible to toxic agents than when it contains either more or less water. Other lines of evidence indicate that this is a phenomenon characteristic of bacterial systems. This principle underlies the lyophilization process for preparing dried microorganisms in a viable condition. If water is removed from such cells at room temperature, the culture dies with great rapidity. Hence, in order to avoid destruction the bacteria are first cooled to a temperature so low that the rate of this lethal process is greatly diminished. Then the water is removed as rapidly as possible. Once dehydrated beyond the critical region, the cells can again be warmed to room temperature without extensive mortality. Application of these considerations has made it possible to devise a simple method for drying microorganisms without resorting to the low temperatures utilized in the lyophilization process. It has been found that cells may be dehyrated at room temperature without loss of viability provided that the desiccation is so rapid that the stable state is achieved before the lethal processes have had time to operate. These studies will be described in a forthcoming publication (14).

The greatly increased efficacy of steam sterilization over dry heating doubtless stems from the same phenomenon. Microorganisms heated in the presence

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fied vapor, which is unity for water; p_o = partial pressure of water vapor in the atmosphere, which would be equal to the product of the atmospheric relative humidity and the vapor pressure of water at the temperature of the experiment; and p_o' = pressure of water vapor in equilibrium with the droplet. For droplets of pure water, p' would be equal to the pure vapor pressure, which is 20.1 mm. Hg. at 22.2°C. If the droplet contains other substances dissolved in the aqueous phase, p' would be lowered by an amount corresponding to the vapor pressure lowering produced by the combined solutes. The presence of salts and proteins within the droplets would decrease the rate of evaporation somewhat but not even a saturated salt solution would lower the vapor pressure sufficiently to decrease this evaporation time by a significant amount.

of steam are maintained in a state of intermediate hydration which makes them more susceptible to the killing action of the high temperatures than would be the case if they were allowed to dry out completely. Still another example of the operation of this same effect may be found in the diminished effectiveness with which aerial disinfectants such as the glycols, kill air-borne microorganisms at low relative humidities (5, 6). This effect is partly due to failure of the germicidal vapor to condense as readily on the desiccated particles which are produced when a bacterial cloud is dispersed in a very dry atmosphere. However, recent experiments *in vitro* have also demonstrated that completely dehydrated bacteria are more resistant to the killing action of the glycols in the liquid state (15).

The values for K_1 and K_2 as here calculated represent the logarithmic rates of removal of viable bacteria from the atmosphere, and so include both the settling rates and the lethal process due to the presence of NaCl at the critical atmospheric humidity. When the appropriate settling rates obtained from Fig. 5 are subtracted from these, extremely small values are obtained for the mortality rates at both very low and very high relative humidities for the three microorganisms tested here. In some cases, the result is negative, which simply means the death rate is too small to be evaluated accurately by this technique.

The nature of the lethal action exercised by NaCl at the critical levels of cellular dehydration probably involves denaturation of one or more essential enzyme systems. Further study of the mechanism of this killing process and of the behavior of bacterial cells in varying states of hydration is being carried out.

Evaluation of the significance of the results reported here for epidemiological problems must await further study. It will be necessary to test in more detail the behavior of a larger number of pathogenic agents, particularly those of the virus group. It is possible that these effects may help to explain certain features of the seasonal pattern of spread of some respiratory diseases. The possibility also exists that humidity regulation by itself might prove to have some effect as a prophylactic measure. Preliminary experiments have shown that by the introduction of a carefully regulated amount of steam into the air of a chamber it is possible to rehumidify and produce rapid and extensive killing of an aerial dispersion of pneumococci which have been allowed to become highly desiccated by an hour's sojourn in an extremely dry atmosphere. While the raising of the relative humidity to the 50 per cent point may not reduce aerial contamination to the same extent as can be achieved by glycol vapors or ultraviolet irradiation, the simplicity of this measure would commend its use in a large variety of situations if it could be shown to exercise even a partially beneficial effect.

SUMMARY

The viability of pneumococcus, Type I, sprayed into the atmosphere from a liquid suspension was measured as a function of the relative humidity. When broth, saliva, or 0.5 per cent saline solution is employed as the suspending medium, a very high mortality rate is observed at relative humidities in the vicinity of 50 per cent. However, at humidities above or below this value the microorganisms survive for long periods.

Measurement of the rate of settling of droplets employed in these experiments demonstrated that the disappearance of microorganisms from the air is a true lethal process, rather than a manifestation of aerosol collision processes.

When a saline-free fluid was used, the sharp peak in death rate at intermediate relative humidities disappears.

The lethal effect of intermediate relative humidities on pneumococci atomized from a saline-containing suspension is increased when the particle size of the atomized droplets is increased or when the temperature is raised.

Cultures of hemolytic streptococcus group C and staphylococcus sprayed from a broth medium exhibit the same general survival pattern as a function of relative humidity although the mortality rates are smaller than that of the pneumococcus.

These effects can be explained by assuming the existence of a critical degree of cellular dehydration at which microorganisms become much more sensitive to toxic agents than in states where either more or less water is bound to the cell.

The results presented here may be significant in elucidating certain aspects of the epidemiology of air-borne infections.

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NUTRITION OF THE HOST AND NATURAL RESISTANCE TO INFECTION

III. The Conditions Necessary for the Maximal Effect of Diet

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What are the requisites for a demonstration of the influence of diet on an infection? Our preceding inquiries (1, 2), using a "natural" disease in a "natural" host, *Salmonella* infection in *Mus musculus*, demonstrated that there were two necessary conditions which had to be fulfilled in the laboratory set-up employed before the outcome of an infection could be measurably influenced by diet. These conditions were (*a*) genetic heterogeneity in the host genotype (1), and (*b*) an array of variation, in terms of virulence, among the pathogen population introduced (2). Given these conditions more infected animals survived on a diet of natural foodstuffs, whole wheat, and whole dried milk, than on an otherwise nutritionally adequate diet composed of purified and synthetic materials, a so called "synthetic" diet (1, 2). Moreover, the indications were that the increased survivorship[1] was due to a certain nutrient, for the effective increase in survivorship was shown to be transferable by the addition of whole wheat to the synthetic diet. Whole dried milk additions did not produce such an effect (1, 2). The nature of the factor present in whole wheat remains unknown.

Foremost among the technological requirements for the identification of a qualitative factor in nutritional experimentation is a revealing method of assay. In the instance here under consideration a statistical increase in survivorship was the sole index of activity available. The biological bases for the statistical character of this index have been discussed previously (1–3). Obviously the assay method should be such that the response in increased survivorship would be maximal and the statistical demands consequently minimal. Experiments which have led to the choices for this arrangement of the laboratory infection model are the subject of the present paper.

[1] It has been found useful to employ "survivorship" to denote the state of being a survivor of an infection. It is this state of surviving without further jeopardy from the infection which is the basic issue at stake in our inquiries rather than the more temporary extension of the duration of life implied by "survival." Survivorship, as an attribute of a population, is measured here by the per cent of survivors, following an infection, after a time interval long enough (30 days) to indicate no further significant change in this statistic. "Increased survivorship" thus means an increased statistical number of survivors.

Materials and Methods

Animals.—The animal stock used was a strain of W-Swiss mice, the same employed in previous investigations (1, 2). The program of outbreeding, previously described (2), has been continued and the stock is free from *Salmonella*.

Pathogen.—Two cultures of *Salmonella typhimurium* (IV, V, XII: i-1, 2, 3) used previously (2) and obtained originally from Dr. G. M. Mackenzie, of the Mary Imogene Bassett Hospital of Cooperstown, New York, were used. One culture, TMO, is relatively avirulent for uniformly susceptible mice and the other, BA_2, is highly virulent. The origin of these strains has been described by Mackenzie, Fitzgerald, and Pike (4). Although the strains differ widely in virulence, they are both smooth, are indistinguishable in cultural, serological, immunizing, and toxigenic characters, and do not differ significantly in invasiveness and resistance to phagocytosis (Pike and Mackenzie (5)). All cultures were perpetuated by preparing nutrient agar stabs with broth cultures of the organisms, incubating for 24 hours at 37.5°C., and then storing in the ice chest at 4°C. Fresh transfers were made at approximately monthly intervals.

Two techniques of infection were used in the current investigation. In one already described (2) the mice, individually caged and with food and water withheld during the preceding 18 hours, drank a 0.25 ml. saline suspension of the bacteria from shallow artist's glazed palette cups. The second method was intraperitoneal injection of the bacteria, in 0.25 ml. of saline. The method used will be stated in each protocol.

Environment.—All of the experiments have been performed in the two air-conditioned rooms previously described (1). These rooms provide a constant temperature of 80° ± 0.5°F. and a constant relative humidity of 50 ± 3 per cent. The artificial lighting (fluorescent) is 12 hours per day, 6 a.m. to 6 p.m.

Diet.—The dietary effect on natural resistance which has been studied here is the difference in the percentageof surviving W-Swiss mice when infected while on a diet of natural foodstuffs (diet 100) and a "synthetic" diet (diet 191) respectively. Previous investigation (1) had shown a higher survivorship (approximately 25 per cent) in the case of the natural diet.

The composition of these diets is as follows:

Diet 100—"Natural Diet"

	gm.
Ground whole wheat	66
Dried whole milk	33
NaCl	1
	100

Diet 191—"Synthetic Diet"

	gm.	mg.
Casein (Labco, vitamin-free)	18.0	
Glucose (cerelose)	72.55	
Salts W-2*	4.0	
l-Cystine	0.2	
Water-soluble vitamins	0.25	
Thiamine hydrochloride		2.5
Riboflavin		5.0
Pyridoxine hydrochloride		2.5

* For composition of salts W-2 see reference 1.

	mg.	gm.
Ca pantothenate	10.0	
Niacin	25.0	
Choline chloride	100.0	
Para-aminobenzoic acid	5.0	
Inositol	100.0	
	250.0	
Fat-soluble vitamins, in cottonseed oil (Wesson)		5.0
	mg.	
β-carotene	0.72	
Viosterol (170 i.u.)		
2-Methyl-1,4-naphthohydroquinone diacetate	0.33	
α-Tocopherol acetate	11.7	
Total		100.0

Both diets: Distilled water *ad libitum*.

Under the environmental conditions already described the mortality risk of uninfected mice on either of these diets is nil. For growth records of W-Swiss mice on these two diets, see reference 1.

Statistical Methods.—The statistical validity of differences in survivorship has been evaluated by application of the χ^2 test. In those instances in which a fourfold table has been applicable the correction of Yates has been introduced.

The Components of the Infection Model

In the assembly of an infection model suitable for the study of the influence of nutrition on survivorship following bacterial infection it has been advantageous to settle on a choice of the W-Swiss strain of laboratory mouse for the host component (1). This choice provided a suitable supply of mice of a satisfactory degree of genetic heterogeneity and without experience with the pathogen to be used, the genus *Salmonella*.[2] As concerns the second component of the infection model, the pathogen population, findings already reported (2) indicated that therein lay possibilities of increasing the dietary effect on survivorship, sharpening, so to speak, the tool of our investigations. If, as has been shown (2), the dietary effect on survivorship is dependent upon a varied array of virulence among the pathogen population, then one might depend on some "stable" culture of *Salmonella* in which a distribution of degrees of virulence exists. But in so doing we would remain ignorant of possible shifts in the frequency distribution of the components and hence our experiments would remain at the mercy of factors not under direct control. Natural populations of *Salmonella* are probably composed of statistical distributions of such components over a more or less continuous spectrum of virulence; for it is a common finding in bacteriology to encounter considerable variation in the virulence of pathogen populations upon initial isolation from the natural infection. It appeared desirable, therefore, to arrange for the compound

[2] The use of "wild" house mice as a more "natural" host, and containing, presumably, a greater degree of genetic heterogeneity will be treated in a separate publication.

nature of the test pathogen population by assembling it from two stable populations of the same species of *Salmonella* which differed widely in virulence. Two stable *S. typhimurium* cultures, the avirulent TMO and virulent BA_2 cultures of Mackenzie (4, 5) were chosen. Still another reason dictated this choice. Separate control of each of the population components offered the possibility of determining by dosage the relationship between the two; for if the important event in the dietary effect on survivorship was dependent qualitatively upon the presence of the two population components, then the quantitative nature of the survivorship difference, not inconceivably, might rest on the numerical relationship between the two. This seemed to offer the possibility of enhancing the survivorship difference due to diet. Operations of this sort though they do not mimic natural events of disease, provide abstractions of them in a form suitable for study, and thus overcome the confining consequences on the dietary effect both of the evolution and domestication of the mouse host and the artificiality of laboratory cultivation of the bacterial pathogen (see (3)).

The Unstable Homogeneity of Virulent S. typhimurium

The relative avirulence of the TMO strain of *S. typhimurium* in broth culture has proved practically unvarying both in Mackenzie's laboratory and our own. In utilizing Mackenzie's virulent BA_2 strain, however, in order to eliminate the heterogeneity which it demonstrably contained (2), a fresh culture (BA_2-SC–1) had been derived from a single colony picked from dilution plates. This culture had proved to be virulent and produced identical mortality rates in W-Swiss mice prepared either on the natural (diet 100) or the "synthetic" diet (diet 191) (2). The question arose, however, whether the presumptive homogeneity of the newly selected and virulent BA_2SC–1 would persist, or whether, as seemed probable, bacterial variation would spontaneously arise, with a consequent rise in survivorship (since the culture would be more likely to vary in the direction of avirulence) and the emergence of a dietary difference in survivorship due to the reestablished heterogeneity. This possibility of the spontaneous rise of variation in virulence during storage of a homogeneous culture was tested in the following experiments.

On three separate occasions samples of 100 weanling W-Swiss mice, 3 to 4 weeks of age, were taken from the outbred W-Swiss colony and divided equally, by litter and sex, into two groups of 50. The animals were transferred to air-conditioned Room 1 and housed in groups of 12 or 13 in the standard monel metal boxes containing purified filter paper pulp as bedding. One group was fed diet 100 *ad libitum*, the second group diet 191, for a period of 3 weeks. The animals were then removed to individual cages in air-conditioned Room 2 and food and water withheld for 18 hours. At the time of transfer to Room 2 the mice had the following average body weights: diet 100 males, 23 gm.; females, 20 gm.; diet 191 males, 20 gm.; females, 18 gm. The mice then were offered 0.25 ml. of a saline suspension of 10^5 cells of an 18 hour broth culture of BA_2SC–1 which had been seeded, in the first experiment, directly from the colony picked

from a dilution plate of the parent BA_2 culture, and in the second and third experiments from an agar stab prepared from this first BA_2SC–1 culture and which had been held in the ice chest at 4°C. for 22 and 36 days respectively. Dosage was adjusted in each experiment by means of a calibrated photoelectric densitometer and was accurate to within 10 per cent as checked by dilution plates poured at the time of infection.

Three hours after the cultures had been offered the animals the infection period was terminated and those which had not consumed the dose were discarded. The others were again supplied with the appropriate diet and water and observed for 30 days. Deaths were recorded daily.

The results of the experiments are set forth in Table I. The data indicate that, with time, variation in virulence arose spontaneously, and after storage of the seed culture at 4°C. for 36 days a prepared broth culture derived from

TABLE I

Spontaneous Changes in a Stored (4°C.) Culture of S. typhimurium Derived from a Single Colony, as Revealed by the Changing Diet Effect on Survivorship

Experiment No.	Date	Time stored	30 day survival, W-Swiss mice*				Survivorship difference	P
			Diet 100		Diet 191			
			S/I‡	%S§	S/I	%S		
		days					*per cent*	
181	3/27/46	0	31/50	62	28/48	58	4	>0.8
184	4/18/46	22	35/48	73	28/46	61	12	>0.3
186	5/ 2/46	36	44/50	88	33/49	67.3	20.7	<0.05

* 100,000 cells, *per os.*

‡ S/I, survivors/infected in test.

§ %S, per cent survivors.

it had enough variation to make possible increased survivorship on diet 100, an increase of 26 per cent ($P < 0.01$) and the emergence of a dietary difference effect on survivorship between diet 100 and diet 191 of 20.7 per cent ($P < 0.05$). It was evident that it would be difficult to preserve a homogeneous, virulent culture of BA_2, and prepare subcultures for use, without the gradual rise of variation in virulence, at least by these methods.

If the spontaneous rise of variation in virulence defeats the aim of laboratory perpetuation and the preparation therefrom of cultures of *S. typhimurium* homogeneous in virulence then, it next occurred to us, perhaps such uniform cultures might be prepared, each time one was required, by using directly as seed the presumptively uniform cells of a single colony of the original BA_2 culture as presented by the fresh preparation of a dilution plate. This notion was put to trial:—

On five separate occasions samples of 100 weanling W-Swiss mice, 3 to 4 weeks of age, were taken from the outbred W-Swiss colony and divided equally, by litter and sex, into two groups

of 50. One group was fed diet 100 *ad libitum*, the second group diet 191 for a period of 3 weeks. Housing and care were as previously described as was the subsequent *per os* infection, observation, and recording of deaths for the postinfection period of 30 days.

Five different cultures of *S. typhimurium*, BA_2, were used in these five experiments. These were, respectively, an 18 hour broth culture of the parent BA_2 population, and four 18 hour broth subcultures seeded directly from single colonies picked at random from dilution plates of the parent BA_2 population which had been poured and incubated in the 18 hours just preceding each such picking. Dosage in all instances was 100,000 viable cells, by mouth.

The results of the five experiments are presented in Table II. It is evident that the parent BA_2 *Salmonella* population was heterogeneous with respect to virulence, for there occurred not only a definite dietary difference in survivorship (24 per cent, $P < 0.05$) but the four single colony cultures picked from

TABLE II

Virulence Differences among Single Colony Cultures Derived from a Parent Culture of S. typhimurium (BA_2) as Measured by Survivorship of W-Swiss Mice Reared on Two Different Diets

Experiment No.	Date	Culture tested, *per os* (dose, 10^5)	30 day survival				Dietary difference	P
			Diet 100		Diet 191			
			S/I*	%S‡	S/I	%S		
							per cent	
180	3/ 8/46	Parent BA_2	27/50	54	15/50	30	24	<0.05
181	3/27/46	Single colony 1	31/50	62	28/48	58	4	>0.8
189	6/13/46	" " 2	35/49	71.4	23/48	47.9	23.5	<0.05
191	6/19/46	" " 3	48/50	96	36/40	90	6	>0.3
192	6/26/46	" " 4	45/50	90	39/50	78	12	>0.1

* S/I, survivors/infected in test.

‡ %S, per cent survivors.

this culture at random exhibited differences in survivorship among the mice, for example, on diet 100, greater than can be attributed to chance ($P < 0.001$). That these differences were attributable to differences among the cultures used, not to the mice, can be safely inferred from an analysis of the sampling methods used in these experiments (1).

It should be noted that three out of the four times that single colony cultures were freshly prepared, and were presumptively homogeneous, survivorship differences due to diet were small and statistically insignificant, although in one of these (Experiment 192) the difference came close to the dividing line of statistical significance generally employed ($P = 0.05$). In one of the four experiments with a single colony culture a dietary difference frankly emerged (Experiment 189). This indicated that probably during the multiplication of the *Salmonella* population from the presumptive single cell in the dilution

plate to the final broth culture used in the experiment, a significant degree of variation would take place spontaneously. Since this event could not be prevented it was evident that homogeneous cultures of *Salmonella* could not be prepared with any certainty by the methods outlined.

The Influence on the Dietary Effect of Inoculating Mixtures of Virulent and Avirulent Organisms

Attempts were made next to bring about the interaction of virulent and avirulent *Salmonella* in ways in which the heterogeneity of the virulent bacterial population might not be of sufficient importance to allow a dietary effect on survivorship, but in which dietary effects could be brought out by appropriate and controllable introduction of the avirulent bacterial population. If the important event which makes a dietary effect on survivorship possible is the presence of both virulent and avirulent cells in the infecting bacterial culture, then the processes which take place between these virulent and avirulent cells, whatever their nature, begin with their introduction into the host; *i.e.*, at time zero. Introduction of the two populations at different times, which would be merely a manipulation of these relationships, might conceivably lead to new phenomena in which the effect of diet might be enhanced, or at least brought under better control.

As we have now seen, an important requirement for a controllable infection model, from the standpoint of the pathogen, is the ability to provoke with the virulent culture high mortality rates (low survivorship) independent of diet, and low mortality rates (high survivorship), independent of diet, with the avirulent culture. The dietary effect on survivorship, is then dependent on, and hence controlled by, the establishment of a relationship between the two cultures in the same host. The important elements of this relationship are number (dose) and time. In the following experiments the interaction of various doses of both virulent and avirulent *Salmonella typhimurium* was tested for two time intervals, the avirulent culture preceding the virulent one by 24 and 72 hours.

On two separate occasions 300 weanling W-Swiss mice, 3 to 4 weeks of age, were taken from the outbred W-Swiss colony and divided, by litter and sex, into thirty groups of 10 mice, each group containing 5 males and 5 females The odd-numbered groups were fed diet 100 and the even-numbered groups were fed diet 191. The animals were housed and cared for as usual for a period of 3 weeks, when they were removed to the infection room (Room 2) and individually caged.

For infection purposes an intraperitoneal injection method was selected in order to (*a*) control the time of infection more accurately than feeding afforded, and (*b*) increase attack rates to 100 per cent mortality, in certain instances, by overcoming the infective inefficiency of the oral route and thereby avoiding the necessity of introducing large doses of the organism, with the attendant risk of introducing variant cells. This change of route of infection was permissible since Webster (6) had shown that mice naturally resistant to *Salmonella* infection *per os* were similarly resistant when injected intraperitoneally or intravenously.

On each of the two occasions the 300 mice were divided into four lots with both diets equally represented. Lot I consisted of 60 mice, lots II, III, and IV of 80 mice each. Each lot received a different dose, ranging up to 10^5 viable cells, of the avirulent culture, in 0.25 ml. of saline. Each of these lots was then subdivided (see Tables III and IV) into diet pairs of 20 mice, 10 on diet 100 and 10 on diet 191, and after 24 hours (Table III), or 72 hours (Table IV), each pair received the appropriate dose of virulent cells, ranging up to 10^5 cells. In this way the effects of fifteen different dose combinations of virulent and avirulent cells were tested for each of the two time intervals.

TABLE III

The Effect of Diet on Survivorship in W-Swiss Mice Infected with Various Doses of Avirulent S. typhimurium, and Superinfected after 24 Hours with Various Doses of the Virulent Organism

Lot	Initial dose avirulent TMO-S3	Diet	Superinfection dose, 24 hrs., virulent BA_2SC-1							
			0		10^2		10^3		10^4	
			S/I*	Difference‡	S/I	Difference	S/I	Difference	S/I	Difference
				per cent		*per cent*		*per cent*		*per cent*
I	0	100			0/10	0	0/10	0	0/10	0
		191			0/10		0/10		0/10	
II	10^2	100	9/10	−10	5/10	20	0/10	0	0/10	0
		191	10/10		3/10		0/10		0/10	
III	10^3	100	9/10	0	7/10	10	5/10	50	4/10	40
		191	9/10		6/10		0/10		0/10	
IV	10^4	100	5/10	−50	7/10	30	4/10	0	6/10	40
		191	10/10		4/10		4/10		2/10	

* S/I, survivors/infected in test.

‡ Difference in survivorship in per cent, reported as positive in sign where diet 100 survivors exceed diet 191 survivors.

The cultures used were 18 hour broth subcultures prepared from agar stabs of *S. typhimurium* having the following histories. The avirulent culture used was TMO-S3 which had been derived from Mackenzie's TMO by recovery *in toto* from the spleen of a known susceptible mouse (BSVS strain, see (1)) surviving 22 days after infection by mouth. The virulent strain was the BA_2SC–1 described earlier in this paper.

The mice were observed for the usual 30 days after the final infection. Results are reported in Tables III and IV.

Several aspects of the data presented in Tables III and IV merit attention:—

1. It is evident that the single intraperitoneal injection of virulent BA_2-SC–1, ranging in dose from 10^1 through 10^5 resulted in death of all the mice

irrespective of diet. By this route, then, BA_2SC–1 behaved *as if* it were homogeneous in virulence; *i.e.*, it provoked a uniform mortality rate (100 per cent) independent of diet.

2. The single intraperitoneal injection of avirulent TMO-S3, up to doses of 10^4, provoked a uniformly low mortality (10 per cent), independent of diet. Doses of 10^4 and 10^5 showed an increased, widely varied attack rate, which

TABLE IV

The Effect of Diet on Survivorship in W-Swiss Mice Infected with Various Doses of Avirulent S. typhimurium, and Superinfected after 72 Hours with Various Doses of the Virulent Organism

Lot	Initial dose avirulent TMO-S3	Diet	Superinfection dose, 72 hrs., virulent BA_2SC–1							
			0		10^1		10^3		10^5	
			S/I*	Difference‡	S/I	Difference	S/I	Difference	S/I	Difference
				per cent		*per cent*		*per cent*		*per cent*
		100			0/10		0/10		0/10	
I	0					0		0		0
		191			0/10		0/10		0/10	
		100	9/10		10/10		4/10		0/10	
II	10^1			−10		10		40		0
		191	10/10		9/10		0/10		0/10	
		100	10/10		10/10		6/10		3/10	
III	10^3			20		30		30		30
		191	8/10		7/10		3/10		0/10	
		100	5/10		2/10		1/10		0/10	
IV	10^5			50		20		10		0
		191	0/10		0/10		0/10		0/10	

* S/I, survivors/infected in test.

‡ Difference in survivorship in per cent, reported as positive in sign where diet 100 survivors exceed diet 191 survivors.

may be tentatively attributed to variant virulent cells present in unpredictable frequencies in TMO-S3 populations of this size.

3. Although the mouse groups compared on each of the two diets at any one combination of avirulent and virulent doses were too small to permit statistically adequate demonstrations of a dietary effect, it is evident from the data taken as a whole that increasing exposure to the avirulent TMO-S3, either by increasing dosage or by lengthening of the time elapsed before the virulent BA_2SC–1 was introduced, tended to increase survivorship. Moreover, it is apparent that there were dose combinations available in which this

increasing survivorship was greater on diet 100 than on diet 191; *i.e.*, a dietary effect on survivorship emerged. In spite of the statistical inadequacies it is also evident that there was a certain orderliness in the relation of the degree of dietary effect at one dose combination to that for other dose combinations. But more important it is clear that the dietary effect on survivorship was indeed a function of the relationship between avirulent and virulent members of the pathogen population; for various alterations in their numerical relationships, in quantity and in time, revealed a whole spectrum of possibilities ranging from circumstances in which no dietary effect was perceptible, to those in which a dietary effect was plainly to be seen.

One of the dose combinations which gave promise of an increased dietary effect was 10^3 TMO-S3 followed by 10^3 BA_2SC–1 after 24 hours. It was decided to use this dose combination and determine the effect of the time interval with larger samples and over a greater period of time.

360 weanling W-Swiss mice, 3 to 4 weeks of age, were taken from the outbred W-Swiss colony and divided, by litter and sex, into nine groups of 40 each. Half of each group were fed diet 100 and the remaining half diet 191. Housing and care of the mice for the 3 week feeding period were the same as before. After 3 weeks the mice were transferred to individual cages in the infection room and the nine groups were infected. The cultures used were 18 hour broth subcultures of TMO-S3 and BA_2SC–1, both at a dose of 10^3 injected in 0.25 ml. saline, intraperitoneally. Group one received TMO-S3 alone; two, BA_2SC–1 alone; all others received TMO-S3 and at intervals of 0, 1, 2, 4, 7, 14, and 21 days the remaining groups each received the superinfecting dose of BA_2SC–1. The superinfection at 0 days was a single injection of a mixture of TMO-S3 and BA_2SC–1 in the proper dosage. The animals were observed for 30 days after the last infecting dose. Results are presented in Table V and Fig. 1.

The consequences of diet on survivorship after double infection with avirulent and virulent *S. typhimurium* are clearly reflected in the data presented in Table V and, graphically, in Fig. 1. The survivorship which resulted as a consequence of exposure to the avirulent TMO-S3 is revealed as increasing with the duration of this exposure, and the effect of diet finds its expression in the relative rates at which this increase in survivorship is achieved as exhibited upon challenge with the virulent BA_2SC–1 culture. These relationships can be depicted in curves, as in Fig. 1, and the dietary effect, when explored completely from minimal to maximal survival, is obviously represented by the area between the curves. Such curves and the areas between them clearly portray the effect of diet in its dynamic aspects, but the utility of such a diagram has another aspect as well. It is obvious that in all probability the dietary effect is most significant,—for these doses and under these conditions,—when a time interval of 1 day was used. For nutritional assay purposes it appeared that these conditions of dosage and time interval would be optimum for the controlled demonstration of the dietary effect on survivorship. This was tested with larger samples of mice and the 2 day interval included in the test as a further check.

TABLE V

Survivorship of W-Swiss Mice on Diets 100 and 191 after Intraperitoneal Injection of Avirulent TMO-S3, Followed after Varying Time Intervals by Virulent BA_2SC–1

Superinfection interval	Dose TMO-S3	Dose Ba_2SC–1	Diet	S/I*	%S‡	Difference	P
days						*per cent*	
Controls	10^3	—	100	18/20	90	0	—
			191	18/20	90		
	—	10^3	100	0/20	0	0	—
			191	0/20	0		
0	10^3	10^3	100	0/20	0	0	—
			191	0/20	0		
1	10^3	10^3	100	15/20	75	65	<0.001
			191	2/20	10		
2	10^3	10^3	100	14/20	70	50	<0.01
			191	4/20	20		
4	10^3	10^3	100	16/20	80	40	<0.05
			191	8/20	40		
7	10^3	10^3	100	18/20	90	50	<0.01
			191	8/20	40		
14	10^3	10^3	100	16/20	80	20	>0.3
			191	12/20	60		
21	10^3	10^3	100	20/20	100	40	<0.01
			191	12/20	60		

* S/I, survivors/infected in test.

‡ %S, per cent survivors.

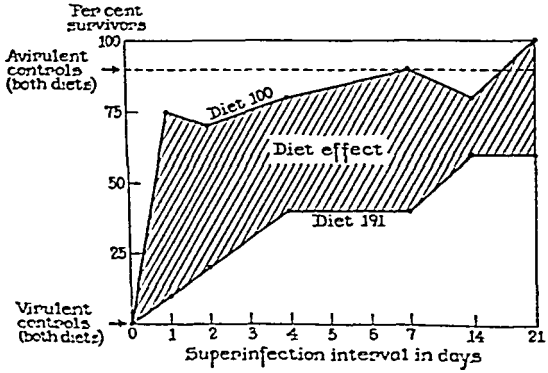

FIG. 1. Survivorship of W-Swiss mice on diets 100 and 191 after intraperitoneal injection of 10^3 avirulent TMO-S3 followed by 10^3 virulent BAS_2C–1 after various times.

200 W-Swiss weanling mice, 3 to 4 weeks of age, were taken from the outbred W-Swiss stock and divided, by litter and sex, into two groups of 100 mice, 50 males and 50 females, each. Half of each group was fed diet 100 and the other half diet 191 for a period of 3 weeks as usual. Upon removal to the infection room all the mice received intraperitoneal injections of 10^3 TMO-S3. Twenty-four hours later one group was challenged with 10^3 BA_2SC–1, and after another 24 hours, the second group was similarly challenged. The mice were then observed for a period of 30 days following the last infection. Results are presented in Table VI.

The results of this experiment clearly indicated that the 24 hour interval was the optimum time interval with a dietary difference in survivorship of 72 per cent. With this final choice of the time interval the infection model for the assay of the factors involved in the survivorship difference effect was now complete. The final test of the infection model was its reproducibility which

TABLE VI

Survivorship of W-Swiss Mice on a Natural Diet (Diet 100) and a Synthetic Diet (Diet 191) after Intraperitoneal Injection of 10^3 Avirulent TMO-S3, Followed after 1 or 2 Days by 10^3 Virulent BA_1SC–1

Superinfection interval	Diet	S/I*	%S‡	Difference	P
days				*per cent*	
1	100	39/50	78	72	<<0.001
	191	3/50	6		
2	100	38/50	76	40	<0.001
	191	18/50	36		

* S/I, survivors/infected in test.

‡ %S, per cent survivors.

depended, of course, on the stability of the bacterial cultures and the frequency of the genotype in the W-Swiss mouse population which was plastic to the nutritional factors being studied. Because of the relatively small size of the breeding W-Swiss stock it must be expected that genetic drifts would, from time to time, increase or decrease the frequency of this plastic genotype. Experience has shown that the infection model is practicable, however, as evidenced by successive tests over a period of 4 months.

On ten occasions, usually as controls for other experiments, 40 W-Swiss weanlings, 3 to 4 weeks of age, were taken from the W-Swiss outbred colony and divided by litter and sex into two groups of 10 males and 10 females each. One group was fed diet 100 and the other diet 191 as usual. After 3 weeks the animals were removed to individual cages in the infection room and injected intraperitoneally with 10^3 of avirulent TMO-S3 and after 24 hours more with 10^3 of virulent BA_2SC–1. Observation was continued for 30 days following the last injection. The results are reported in Table VII.

These ten tests of the infection model demonstrated that a dietary difference in survivorship could be reproduced consistently. This difference was of the order of 55 per cent, which was a considerable increase from the 25 per cent difference established in our first report (1) in which the bacterial population used was a single, presumably heterogeneous strain of *S. enteritidis*. In the present model tests for heterogeneity of the data of Table VII reveal that, considered on the basis of reproducibility of the specific mortality rates on each

TABLE VII

Ten Tests of the Dietary Difference in Survivorship Obtaining between Diet 100 and Diet 191 When W-Swiss Mice Were Infected with 10^3 Avirulent TMO-S3 and, after 24 Hours, Challenged with 10^3 Virulent BA_2SC-1

Test	Date	Diet 100		Diet 191		Frequency of survival difference	Survivorship difference	Tests of heterogeneity	
		S/I*	%S‡	S/I	%S			By attack rate	By survival difference frequency
							per cent	χ^2	χ^2
1	3/12/47	15/20	75	2/20	10	13/20	65	0.8038	0.8080
2	3/26/47	15/20	75	1/20	5	14/20	70	1.4422	1.8181
3	3/26/47	15/20	75	2/20	10	13/20	65	0.8038	0.8080
4	4/17/47	15/20	75	2/20	10	13/20	65	0.8038	0.8080
5	4/29/47	9/20	45	1/20	5	8/20	40	4.3631	1.8181
6	4/29/47	8/20	40	0/20	0	8/20	40	8.1012	1.8181
7	5/15/47	15/20	75	4/20	20	11/20	55	2.7192	0.0000
8	5/15/47	12/20	60	0/20	0	12/20	60	2.6139	0.2020
9	6/17/47	12/20	60	4/20	20	8/20	40	2.1882	1.8181
10	7/ 3/47	15/20	75	5/20	25	10/20	50	5.2731	0.2020
Total.....		131/200		21/200				29.1123	10.1004
Average.....			65.5		10.5		55.0	P <0.001	P >0.3

* S/I, survivors after 30 days/infected in test.

‡ %S, per cent survivors.

of the two diets, deviations occurred which were greater than can be attributed to chance ($\chi^2 = 29.1123$; $n = 9$; $P < 0.001$). But the important event in these experiments is not the specific mortality rates, but the magnitude of the dietary difference between the mortality rates on the two diets. When analyzed from this standpoint (Table VII) it is obvious that the total experience with the infection model, as reported in Table VII, is consistent and homogeneous and the variations in the dietary difference in survival are no more than can be expected on the basis of chance ($\chi^2 = 10.1004$; $n = 9$; $P > 0.3$). With a mean dietary effect of 55 per cent,—or 11 mice out of every

20,—for samples of 20 mice on each diet it may be expected that the dietary difference will only rarely ($P < 0.05$) be any less than 35 per cent. It can be concluded that the model is a satisfactory and reliable means of demonstrating the dietary effect on survivorship following infection with *S. typhimurium*.

DISCUSSION

These experiments, and those preceding (1, 2), have shown that under certain conditions diet influences the outcome of an infection and under others it does not. Thus, in these matters, it has been found that in the laboratory demonstration of a dietary effect on infection those hosts are satisfactory which are genetically heterogeneous and unselected and those pathogen populations are satisfactory which are heterogeneous in their array of variation in virulence. In order to improve the plasticity of the infection model to the effect of diet a study was made, in the present paper, of the interaction in the host in time and in number of both virulent and avirulent *S. typhimurium*. This has led to the devising of an infection procedure involving the curious manipulations of injecting the avirulent culture first and after 24 hours injecting the virulent culture. This is hardly an imitation of a "natural event," for such an event must be a very rare one in the natural world. It must be remembered, however, that in the instance of the present model the 24 hour interval is related only to the establishment of the relationship between the two particular cultures being used. For other avirulent and virulent cultures of *S. typhimurium* a similar analysis might well lead to the choice of other doses and other time intervals. These other doses and these other time intervals would be on more than a reflection of the variation among those representatives we elect to call "virulent" or "avirulent." But the important point is that certain doses and certain time intervals may be found for any combination of cultures at which the diet effect is maximal, for the diet effect rests, not on the special attributes of any one culture, but on the difference which exists between any two. Now, it may be that the difference in virulence between two cultures taken at random from the natural world may be trivial, and hence would hardly serve in experiments such as are described here. Such cultures would be rejected and others sought which were more practicable, but from a theoretical viewpoint the power and general application of this method of assembling a satisfactory infection model would remain. If we hope to learn the chemical nature of the influential nutritional entities, we must so arrange the model as to obtain the widest possible divergence in survivorship in order to obtain a practicable method of assay for active materials. The assay method described in this paper has its basis in a phenomenon which is revealed uniquely by bringing into relationship two different strains of the same bacterium which are divergent in virulence. We propose to name this phenomenon the "double strain phenomenon," and the assay method based on it the "double strain inocula-

tion," or DSI, method. That this analysis of the infection model has something to recommend it can be illustrated by the following comparison. The DSI method can be pursued with samples as few as 10 mice per sample and with which a diet difference as small as 10 per cent will be a rare event ($P < 0.001$). Most of the time, of course, the dietary difference will be greater. If, instead of the DSI method, the infections were performed by the method used in the first paper of this series (1), which involved the feeding of mice with a single culture of *S. enteritidis*, standard statistical calculation[3] shows that at a similar level of 10 per cent difference in diet effect on survivorship it would be necessary to employ samples of 103 mice from the same stock. The expansion of the DSI method to a sample size of 20, which is practicable, renders the assay of the nutritional effect on infection survivorsh p a statistically sound undertaking. Its results will be the subject of future publications.

One more matter merits attention. What is the nature of the relationship in the host of the avirulent and virulent *Salmonella* populations? Does the host participate directly to furnish antibodies under the stimulus of the avirulent bacteria and thus mediate the effects of the virulent cells when they gain admittance? If so, this type of response of the host must be more precocious than is usually the case for, as we have seen, the maximal effect is apparent after 24 hours. Indeed, for other cultures of avirulent and virulent *S. typhimurium* (2) a diet effect can be demonstrated when the two populations are admitted simultaneously. Or, it might be suggested that, on the face of the data, avirulent populations "interfere" with the virulent ones, almost in the sense that certain viruses "interfere" with the effects of others, and that the rate of the establishment of this "interference" is a function of diet. Whatever may be the basis of the diet effect, be it immunologic, "interference," or otherwise, the fact of the interaction of avirulent and virulent *S. typhimurium* as the basis of the dietary effect on survivorship has been demonstrated, and this fact has led to the design of a statistically adequate assay method for the eventual isolation and identification of the nutritional entities involved.

SUMMARY

The observation has been confirmed that the property of a "natural" diet of whole wheat and whole dried milk to promote a higher survival rate among a stock of outbred, genetically heterogeneous W-Swiss mice subjected to *S. typhimurium* infection, over that promoted by a "synthetic" diet, is dependent upon a relationship between avirulent and virulent members of the pathogen population.

This relationship has now been analyzed in terms of number (interacting doses) and time (interval between interacting doses). On any given diet

[3] These calculations have followed the methods outlined by Mather in his Statistical Analysis in Biology, New York, Interscience Publishers, Inc., 1947.

survivorship has been demonstrated to be a function of the host's experience of the avirulent pathogen; increasing experience, either in dose or time, resulted in increasing survivorship. For a given set of interacting avirulent-virulent doses the rate at which survivorship rose was greater when the "natural" diet was fed than when the "synthetic" diet was fed. This difference in survivorship-increase rates gave rise to differences in survivorship between the two diets. These dietary differences ranged from minimal to maximal with increasing time, and then, as still more time elapsed, back to minimal again. In consequence an optimum time interval was found at which the dietary effect was maximal. This time interval was chosen for assay purposes. The assay method thus arrived at was tested repeatedly for reproducibility, and statistical analysis showed it to be reliable and capable of demonstrating a mean dietary difference in survivorship of 55 per cent.

The nature of the relationship established between avirulent and virulent *S. typhimurium* in mice has been discussed.

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EXPERIMENTS ON THE RÔLE OF THE CHICKEN MITE, DERMANYSSUS GALLINAE, AND THE MOSQUITO IN THE EPIDEMIOLOGY OF ST. LOUIS ENCEPHALITIS*

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Epidemics of acute encephalitis occurred in the St. Louis area in 1933 and 1937 (1,2). The distribution of cases (3) and the summer incidence of the disease suggested the possibility of a blood-sucking vector. In 1933 two independent groups of investigators, one from the United States Public Health Service and the other from the United States Army, undertook studies concerning possible implication of mosquitoes. Results of their experiments were negative (1).

In 1935, however, Webster, Clow, and Bauer (4) showed that *Anopheles quadrimaculatus* could be infected with the virus of St. Louis encephalitis. Such mosquitoes shown to harbor the virus for from 21 to 42 days did not infect mice or monkeys by bite. In 1937, Fulton, Greutter, Muether, Hauss, and Broun (5) were successful in infecting *Culex pipiens* with the virus of St. Louis encephalitis by allowing them to feed on infected mice. The virus did not survive in the bodies of these mosquitoes for longer than 10 days, and normal mice could not be infected by their bite. Mitamura and his associates (6) reported in 1937 successful transmission of the virus of St. Louis encephalitis by *Culex pipiens* var. *pallens* Coq. and by *Aëdes logoi*.

In 1941, Blattner and Heys showed that an arachnid, the dog tick (*Dermacentor variabilis*), could be infected with the virus of St. Louis encephalitis and could transmit the virus to mice by bite (7). Subsequent investigations (8) revealed that under experimental conditions this tick is capable of transferring the virus to susceptible animals by bite in any stage of its life cycle and of passing the virus to its offspring through the egg. Ticks which had been kept for 10 months at a temperature of 12.5°C. retained virus during hibernation and were shown to transmit the infection to animals by bite. Likewise eggs laid by infected females retained virus over the winter, and larvae hatched from these eggs harbored virus and were capable of infecting susceptible animals. Thus for the first time it was demonstrated that transovarial or congenital passage of this virus in an arachnid occurs under experimental conditions.

In 1942, Reeves, Hammon, and Izumi (9) reported transmission of the virus of St. Louis encephalitis by *Culex pipiens* Linn. Subsequently Hammon and Reeves were able to show that mosquitoes belonging to a number of genera are capable of acquiring

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the virus and of transmitting the virus to animals (10). Hammon, Reeves, and their associates (11) isolated the virus of St. Louis encephalitis from culicine mosquitoes collected in nature during epidemic periods in the Yakima Valley in Washington.

The virus has been shown to remain in the body of the mosquito for 28 days (10) and for 42 days (4). Transovarial passage of the virus in the mosquito, however, has not been demonstrated, and to our knowledge the virus has not been detected in hibernating mosquitoes. In consequence it seems unlikely that the mosquito could be the sole agent concerned in the epidemiology of St. Louis encephalitis.

That an endemic focus exists in the St. Louis area since the epidemics of 1933 and 1937, is indicated by the fact that each summer cases of acute encephalitis have been proved by appropriate neutralization tests to be of the St. Louis type (12). A serum-neutralization survey was made in the St. Louis area in 1943 and 1944 (13). In St. Louis County where endemic cases of St. Louis encephalitis have been shown to occur antibody for the St. Louis virus was demonstrated in sera of chickens from certain flocks. Antibody of low titer was present in 11 to 33 per cent of chickens chosen from 5 flocks. Demonstration of antibody was equivocal in an additional 12 to 14 per cent of chickens in these same flocks. Of 97 people tested who had resided in St. Louis County through the epidemic years of 1933 and 1937, 37 per cent showed antibody to the St. Louis virus. In certain sections of St. Louis County the sera of approximately 50 per cent of residents tested contained antibody. On the other hand, only a few (3 of 56) people (children 3 to 7 years of age and adults who had come from states east of the Mississippi since 1937) who had resided in St. Louis County since 1937 showed antibody. In view of these results it seemed logical to conclude that some vector in the St. Louis area, which as a rule does not attack human beings, feeds upon chickens, and thereby introduces virus into their bodies. The chicken mite, *Dermanyssus gallinae*, an arachnid similar biologically to the dog tick, seemed a likely possibility. Accordingly, collections of this chicken mite were made in selected districts of St. Louis County. Chicken mites from 3 different sites, 6 to 10 miles distant, yielded an infectious agent which in each case was identified as the virus of St. Louis encephalitis (14).

In previous reports from this laboratory (14, 15) evidence was presented that the chicken mite (*Dermanyssus gallinae*) is capable of transferring the virus of St. Louis encephalitis congenitally to its offspring *ad infinitum*, and that a colony of mites once infected probably remains infected indefinitely. Uninfected mites derived from a single female and her nymph offspring and shown to be free of virus could be infected readily by feeding on animals inoculated with various strains of the virus of St. Louis encephalitis. Experimentally infected mites as well as those found infected in nature proved capable of transferring the virus through the egg to their offspring. Both naturally infected and experimentally infected mites transferred the virus to chickens by bite. Uninfected mites could acquire virus from chickens bitten by infected mites. Viremia in chickens bitten by such mites was demonstrated with regularity by the use of the chorioallantoic passage and subsequent intracerebral inoculation of mice (16). Isolation of the St. Louis encephalitis virus from mites in nature and

results of laboratory experiments indicating maintenance of the virus in mites suggest that the mite may serve as a reservoir in nature. Likewise the fact that virus is present in the blood of chickens following the bite of infected mites suggests the possibility that during the period of viremia such chickens might serve as a source of virus for other blood-sucking vectors, possibly the mosquito, of which several species are known to feed upon chickens (17).

The present report concerns experimental work which indicates that chickens acquiring the virus of St. Louis encephalitis from infected mites have sufficient virus in their blood during the period of viremia for mosquitoes to acquire the virus while feeding, and that such infected mosquitoes are capable of transmitting the virus to other animals.

Methods and Materials

Species of Mosquitoes.—Seven species of mosquitoes, belonging to 3 genera, used in the present investigations, were obtained from various sources: *Culex pipiens* Linn., collected as eggs, larvae, and pupae in St. Louis County and maintained as a breeding colony at Washington University; *Culex quinquefasciatus* Say, collected as eggs, larvae, and pupae in New Orleans and maintained as a breeding colony at Washington University; *Anopheles punctipennis* (Say), collected as larvae in St. Louis and vicinity; *Anopheles quadrimaculatus* Say, from eggs obtained from a colony at Tulane University (an Alabama strain) and also from eggs of females captured and from larvae collected in St. Louis and vicinity; *Aëdes aegypti* (Linn.), from eggs obtained from a colony at Tulane University (a New Orleans strain) and maintained as a breeding colony at Washington University; *Aëdes triseriatus* (Say), collected as larvae in the vicinity of St. Louis; and *Aëdes vexans* (Meig.), collected as larvae and pupae and also from eggs laid by females captured in St. Louis and vicinity.

Rearing of Mosquitoes.—All mosquitoes used in the experiments were reared in the laboratory from preadult stages. A mosquito-proof, air-conditioned room having a two doored vestibule and sealed windows was provided. A closed circuit of air was maintained by a specially designed humidifier communicating with the room. Air was drawn through excelsior-packed screens kept wet by dripping water. Manual adjustment of the flow and the temperature of the water controlled the rate of evaporation, providing the desired humidity and reducing temperature fluctuation. The temperature in the breeding room varied from 75° to 91°F. and the relative humidity from 85 to 92 per cent. This room was used only for the production of mosquitoes. No infected animals were permitted in the breeding room. Food for the adult mosquitoes in the breeding cages consisted of dextrose solution, cut prunes, and blood of uninfected host animals. For the species of *Culex*, young chickens were used as hosts and were kept in the breeding cages continuously. These chickens had been hatched in the laboratory and had been protected at all times from possible exposure to arthropods by fine wire screen and a surrounding moat of cresol. A guinea pig was used as host for *Aëdes aegypti* and a rabbit for *Anopheles quadrimaculatus*, the animals being placed in the cages for several hours each day. Eggs laid in a small bowl of water (*Culex*) or on wet filter paper (*Aëdes* and *Anopheles*) were transferred to larval breeding pans. Culicine larvae were fed with a suspension of brewer's yeast and dried beef blood albumen, added to the water once or twice during development; anopheline larvae received ground Purina dog chow daily. Pupae removed from breeding pans were placed in metal cups (50 to 150 in each cup) under lamp chimneys which were capped at the upper end with bobbinet mosquito netting. The rubber bands securing the netting also held a piece of filter paper suspended within the chim-

ney to provide additional resting space for the mosquitoes. When all adults had emerged from the pupae (2nd or 3rd day), the containers were removed from the cups, closed below by netting, and placed on wet cellucotton in Petri dishes. Pledgets of cotton saturated with dextrose solution were laid on the top netting to provide food. Mosquitoes were stored in this manner for at least 24 hours before exposure to experimental animals. Thus a constant supply of suitable mosquitoes was available for experimental use.

Infection of Mosquitoes.—A second mosquito-proof room in which the mosquitoes were infected was fitted with a double vestibule closed by three mosquito-proof doors and contained a large screened enclosure with its own two doored entry. This room was air-conditioned in the same manner as the breeding room. The temperature ranged from 72° to 94°F. and the relative humidity between 77 and 96 per cent, the usual range for temperature being 80° to 90°F. and that for relative humidity between 80 and 90 per cent. In the screened enclosure mosquitoes were allowed to feed upon infected animals, and the fed mosquitoes were transferred to clean lamp chimneys. Here the mosquitoes were stored for incubation of virus and later allowed to feed upon uninfected animals for possible transmission of the virus.

Mosquitoes were infected by allowing them to feed upon chickens having viremia or on a suspension of infected mouse brain tissue in broth and defibrinated rabbit blood.

The chickens used as a source of virus for mosquitoes were laboratory bred New Hampshire Reds, 10 to 20 days of age. Viremia was produced in these chickens by the subcutaneous inoculation of the Hubbard egg membrane strain of virus (0.2 ml. of a 1:100 dilution of infected egg membrane in broth) or by the bite of infected mites. Four colonies of infected mites were used for this purpose: the Rippy colony derived from mites found infected in nature and 3 colonies of experimentally infected mites. The latter were infected with 3 strains of St. Louis virus: (*a*) the Rippy strain (RN_6), a mouse brain virus isolated from mites found infected in nature, (*b*) the Hubbard egg membrane strain, an egg membrane strain of St. Louis virus (Hubbard) isolated in mice from human brain tissue in 1937 and maintained since 1938 on the chorioallantoic membrane of the developing hen's egg, (*c*) the Mullen strain isolated in mice in 1945 from the blood of a patient who recovered from encephalitis.

Chickens were used as potential sources of virus for mosquitoes between 48 and 92 hours following the inoculation of virus or following the exposure of chickens to infected mites in a manner previously described. This is the period during which viremia was shown to occur most commonly (15, 16). For verification of viremia in chickens, blood samples drawn before and, in most instances, after the mosquitoes had fed, were tested for virus in the following manner. Heparinized whole blood (0.08 to 0.1 ml.) was inoculated on the chorioallantoic membrane of the developing hen's egg. Four days after inoculation these membranes were harvested and ground with a small amount of tryptose phosphate broth. The resulting suspension was centrifuged at low speed for 2 minutes, and the supernatant fluid was passed to a second series of embryonated eggs. As before, the passage membranes were harvested after a 4 day period and were ground with broth. After centrifugation, the supernatant fluid was injected intracerebrally, in 0.03 ml. amounts, into white Swiss mice.

Mosquitoes to be infected by feeding upon chickens during viremia were allowed access to the unanesthetized chicken which was held by an oilcloth restrainer against the netting of the lamp chimney container. Before the chickens that had been infected by mites were used in this way for the infection of mosquitoes, any mites remaining on their bodies were destroyed by a 5 minute exposure to chloroform vapor. The body of the chicken was placed in a jar covered by a sheet of rubber through which the head protruded. As precaution against possible escape of mites remaining alive, a petrolatum barrier was placed around the base of the stand used to support the lamp chimney during the exposure of the chicken to mosquitoes. The exposure periods varied from 15 minutes to 12 hours, usually overnight, according to

need or convenience. At the close of the feeding period, mosquitoes which contained visible ingested blood were separated from the unfed mosquitoes and placed in other containers. Transfer of unanesthetized mosquitoes by means of an aspirator tube was carried out within a glass-topped box provided with armholes and sleeves of netting.

Mosquitoes to be infected by feeding on a suspension of virus were exposed to the suspension for periods up to 2½ hours. Lamp chimneys containing the mosquitoes were placed on pieces of cellucotton saturated with the virus suspension, the mosquitoes feeding readily through the netting. The virus suspension was prepared at the beginning of the feeding period by adding 1 part of a 20 per cent suspension of infected mouse brain tissue in broth to 6 parts of defibrinated rabbit blood. The brain tissue was that of mice infected with the RN_6 strain of St. Louis virus (isolated from mites collected in nature). The suspension in broth and defibrinated blood prepared at the beginning of the experiment was kept in the refrigerator, and fresh refrigerated suspension was added to the cellucotton at 30 minute intervals during the feeding period.

Mosquitoes that had ingested infective blood or virus suspension were stored in the lamp chimneys for varying periods to allow incubation of the virus. A piece of wire screening suspended in the container provided a resting surface for the mosquitoes. Water was provided through wicks in contact with the bottom netting, and raisins or cotton saturated with dextrose solution were placed on the top netting to supply food.

Isolation of Virus from Mosquitoes.—Attempts were made to isolate virus from suspensions of mosquitoes at periods varying from 8 to 29 days after the ingestion of infective blood. Samples of mosquitoes, after light chloroform anesthesia and removal of legs and wings, were triturated in tryptose phosphate broth using 0.1 to 0.2 ml. of broth per mosquito. In most instances 0.06 ml. of an uncentrifuged or a centrifuged suspension of mosquitoes was inoculated intraperitoneally into young Swiss mice 7 to 12 days of age. When the more concentrated or uncentrifuged suspensions were used, the mice sometimes died 1 to 2 days following inoculation, apparently as a result of heavy contamination of the inoculum. When convulsions or other signs of illness suggestive of encephalitis were observed, brain tissue from the young mice was passed intracerebrally to 4 to 6 adult Swiss mice. In a small number of experiments filtered suspensions of the mosquitoes were tested for virus by direct intracerebral inoculation of adult Swiss mice and/or by chorioallantoic passage on the developing hen's egg with subsequent intracerebral passage to adult mice.

Method for Demonstrating Transmission of Virus by Mosquitoes to Chickens.—Five to 55 days after an infective blood meal mosquitoes were allowed to feed on normal unanesthetized chickens in the same manner as that described for the initial feeding on infected chickens. These normal chickens ranged in age from 6 to 20 days, the majority being 10 to 12 days of age. Following the feeding period the mosquitoes containing visible ingested blood were counted in order to determine the approximate number of bites that the chicken had received. Since a mosquito may pierce the skin of the host without withdrawing a detectable amount of blood, a few exposed animals were tested for viremia even when no blood was visible in any of the mosquitoes. Most of the chickens exposed to the infected mosquitoes were bled only once for viremia test, on the 2nd or 3rd day (34 to 64 hours) after the feeding period. Eleven chickens were bled 3 times, at intervals between 1 and 4 days after the feeding period. Heparinized whole blood was tested for virus by chorioallantoic passage with subsequent intracerebral passage to adult mice, and in most instances serum from blood drawn simultaneously was inoculated intracerebrally into 4 to 6 adult mice.

Method for Demonstrating Transmission of Virus by Mosquitoes to Mice.—Six to 19 days after the infective meal, mosquitoes were allowed access to young Swiss mice, 6 to 14 days of age, which had been anesthetized by dial. The anesthetized mice were placed on the netting covering the lamp chimney containers and held in place by a second piece of netting. Mice

which received one or more bites as determined by counting the number of engorged mosquitoes after the feeding period, were observed for signs of encephalitis. Some of the young mice thus exposed were killed at 10 and 13 days after they had been bitten. Brain tissue from each of these was passed by intracerebral inoculation to 4 to 6 adult mice. The remaining young mice were observed for 21 days.

Method for Demonstrating Transmission of Virus by Mosquitoes to Hamsters.—Four to 42 days after an infective blood meal, mosquitoes were allowed to feed on young adult Syrian hamsters (*Cricetus auratus*) anesthetized with dial. Hamsters were exposed to infected mosquitoes for several hours or overnight by supporting a lamp chimney container over the animal in such a way that the netting covering one end of the container was in contact with the shaved abdomen. The number of mosquitoes biting a given hamster was determined by counting those which contained visible blood after the feeding period. For viremia test some of the hamsters were bled only once at 48 to 81 hours after the beginning of the period during which the mosquitoes fed; others were bled 2 or more times between 24 and 108 hours. Heparinized whole blood was tested for virus by chorioallantoic passage and subsequent intracerebral inoculation of adult mice. Several of the hamsters were inoculated intracerebrally with aleuronat at a time when viremia might be expected. It was thought that damage to the brain tissue produced in this manner might facilitate invasion of the brain by virus in the blood stream. The hamsters were observed for 2½ months.

Identification of Virus.—Strains of virus isolated from chickens and hamsters having viremia were compared with a known strain of St. Louis virus (Hubbard) and were identified as the St. Louis virus by neutralization in mouse protection tests. Immune rabbit sera used in mouse protection tests were prepared by repeated subcutaneous inoculation of normal rabbits with the Hubbard strain of St. Louis virus. Strains of virus isolated from each of 11 hamsters and 10 chickens having viremia as the result of the bite of infected mosquitoes were identified in this manner. These infected mosquitoes, the bite of which resulted in viremia in chickens and hamsters, had acquired the virus by feeding on chickens bitten by mites infected in nature or experimentally infected with 1 of 3 strains of St. Louis virus.

RESULTS

The primary purpose of the present investigation was to ascertain (1) whether or not mosquitoes can acquire the virus of St. Louis encephalitis by feeding on chickens infected by mites, and (2) whether or not mosquitoes thus infected can transmit the virus to chickens, mice, and hamsters.

Isolation of Virus from the Bodies of Mosquitoes

Before attempting transmission of virus by the mosquito it was advisable to show the presence of virus in the body of the mosquito. Accordingly, attempts were made to isolate virus from mosquitoes presumably infected in one of three ways: (1) mosquitoes fed on a suspension of infected mouse brain tissue in tryptose broth and defibrinated rabbit blood; (2) mosquitoes fed on chickens in which viremia had been produced by subcutaneous inoculation of the Hubbard egg membrane strain of virus; (3) mosquitoes fed on chickens having viremia as the result of the bite of infected mites.

The results of these isolation experiments can be summarized as follows:—

Five different species of mosquitoes (of 3 genera) were tested for virus after ingestion of infected mouse brain material. The bodies of the mosquitoes were triturated in tryptose

broth, 0.1 to 0.2 ml. per mosquito, and the resulting suspension was inoculated intraperitoneally into young Swiss mice. One of these 5 experiments, that with *Anopheles quadrimaculatus*, was unsatisfactory, presumably because the uncentrifuged suspension of 11 mosquitoes was contaminated heavily, and the test animals died within 1 or 2 days. Virus was obtained from each of the other 4 species of mosquitoes by the intraperitoneal inoculation of young Swiss mice. Uncentrifuged suspensions of mosquitoes were used in 3 instances and a centrifuged suspension in one instance These 4 species of mosquitoes were *Anopheles punctipennis* (7 mosquitoes), *Aëdes aegypti* (26 mosquitoes), *Aëdes triseriatus* (3 mosquitoes), and *Culex quinquefasciatus* (4 mosquitoes). The period allowed for incubation of the virus in the mosquitoes ranged from 10 to 15 days. Virus was isolated readily from each of the 4 samples, the young mice which were inoculated intraperitoneally developing definite signs of encephalitis in 4 to 9 days. Brain tissue from one or more young mice of each test group was passed to 4 adult Swiss mice by intracerebral inoculation. These inocula proved bacteriologically sterile on culture. The passage mice developed convulsions in 2 to 4 days following inoculations.

Mosquitoes of 3 species (*Aëdes aegypti*, *Culex quinquefasciatus*, and *Culex pipiens*) were tested for virus after being allowed to feed on chickens inoculated subcutaneously with the Hubbard egg membrane strain of virus. Only mosquitoes which had ingested blood were tested for virus. Each chicken used in this way as a source of virus for mosquitoes was shown to have viremia by the direct intracerebral inoculation of mice with serum, as well as by chorioallantoic passage with subsequent intracerebral inoculation of mice. Two samples of *Aëdes aegypti* consisting of 4 and 6 mosquitoes, one of *Culex quinquefasciatus*, 11 mosquitoes, and 1 sample of *Culex pipiens*, 13 mosquitoes, were tested for virus by intraperitoneal or intracerebral inoculation of suspensions into young mice. Owing to contamination 1 sample of *Aëdes aegypti* and 1 of *Culex pipiens* were not satisfactory. Consequently only 2 satisfactory tests were carried out using mosquitoes which had fed on chickens infected by subcutaneous inoculation of virus: 1 with *Aëdes aegypti* and 1 with *Culex quinquefasciatus*. The period allowed for incubation of the virus in mosquitoes in these two instances was 8 and 16 days respectively. A centrifuged suspension of the sample of *Aëdes aegypti* was inoculated intraperitoneally into young mice, and a filtered suspension of the sample of *Culex quinquefasciatus* intracerebrally into adult mice, both giving negative results.

Mosquitoes of 4 species, *Aëdes aegypti*, *Aëdes vexans*, *Culex quinquefasciatus*, and *Culex pipiens*, comprising 13 lots, were tested for virus after feeding on chickens bitten by infected mites. Nine of these 13 lots (3 of *Aëdes aegypti*, 1 of *Aëdes vexans*, 2 of *Culex quinquefasciatus*, and 3 of *Culex pipiens*), were fed on chickens infected by the bite of mites from the Rippy colony, a colony derived from mites found infected in nature. The number of mosquitoes tested varied from 1 to 60. The periods allowed for incubation of the virus in the mosquitoes varied from 13 to 29 days. One of the 9 samples of mosquitoes, an uncentrifuged suspension consisting of 4 *Aëdes aegypti*, was unsatisfactory because of contamination. No virus was isolated from the remaining 8 samples by intraperitoneal inoculation of young mice, none from centrifuged suspensions, and none from uncentrifuged suspensions. A portion of the centrifuged suspension from each of 2 samples, *Culex pipiens*, 60 and 11 mosquitoes respectively, was filtered by means of a Luer-Lok syringe with Swinny adapter. While no virus was isolated from these 2 filtrates by direct intracerebral inoculation of adult mice, the filtrate resulting from 60 mosquitoes which had fed 13 days before, was shown to contain virus by 2 chorioallantoic passages in the developing hen's egg. A suspension of chorioallantoic membrane on transfer intracerebrally to adult mice produced convulsions in 4 days.

One sample of each of 4 lots of *Culex pipiens* which had fed on chickens shown to have viremia as a result of the bite of experimentally infected mites, was tested for virus. The uncentrifuged suspension of 1 sample gave unsatisfactory results when inoculated intraperitoneally into young mice. The centrifuged suspensions of the other 3 samples consisting of

28, 31, and 55 mosquitoes, were filtered by means of the Swinny adapter and tested for virus by the direct intracerebral inoculation of adult mice and simultaneously by the chorioallantoic passage method. Virus was isolated by chorioallantoic passage but not by direct intracerebral inoculation of mice. In these 3 lots of infected mosquitoes, 3 strains of virus were involved: the RN_6, the Hubbard egg membrane, and the Mullen. The periods allowed for incubation of the virus in the mosquitoes ranged from 15 to 21 days.

As the foregoing has shown, virus was detected in suspensions of triturated mosquitoes which had fed on chickens infected by mites by the inoculation of filtered centrifuged suspensions on the chorioallantois of hens' eggs and subsequent intracerebral passage to mice. *Culex pipiens* was the only species of mosquitoes tested for virus in this manner. Virus was demonstrated in these mosquitoes 13 to 21 days following the infective meal. Virus was not isolated from mosquitoes which had fed on chickens infected by mites, either by direct intraperitoneal inoculation of young mice or by direct intracerebral inoculation of adult mice. The same was true for mosquitoes infected by feeding on chickens inoculated subcutaneously with virus suspensions of the Hubbard egg membrane strain. However, virus was isolated readily, by direct inoculation of mice, from mosquitoes of 4 species which had ingested infected mouse brain material 10 to 15 days previously. Where only small amounts of virus are present in the bodies of infected mosquitoes, as in those fed upon chickens infected by mites, chorioallantoic passage increased the amount of virus to an extent such that signs of encephalitis are produced in mice when egg membrane material is inoculated intracerebrally.

Transmission of the Virus to Chickens by Mosquitoes Infected by Feeding on Virus Suspension

Transmission of the St. Louis virus to normal chickens was attempted using mosquitoes of each of 5 species, (*Anopheles punctipennis, Anopheles quadrimaculatus, Aëdes aegypti, Aëdes triseriatus, Culex quinquefasciatus*), which had ingested a suspension of infective mouse brain tissue (RN_6 strain of virus) in broth and defibrinated blood. Following the ingestion of infective material, periods of 8 to 12 days were allowed for incubation of the virus in these mosquitoes. After the incubation period each of the 5 lots of mosquitoes was allowed access to one chicken. Three of the 5 chickens were bitten by 2 mosquitoes each and 2 chickens by only 1 mosquito each, as determined by counting the mosquitoes which had ingested blood. Three tests for viremia were made on each of the 5 chickens, blood being drawn at intervals between 1 and 4 days after exposure to the mosquitoes. Serum was tested for the presence of virus by direct intracerebral inoculation of adult mice. Simultaneously, heparinized whole blood was inoculated on the chorioallantoic membrane. In each of the 5 chickens viremia was detected by chorioallantoic passage and subsequent inoculation of adult mice. Virus was demonstrated in 3 samples of blood from

each of 4 chickens and in 1 sample (37 hour) from the fifth chicken. Results of direct serum inoculation were negative except in one instance in which the result was equivocal. In this instance 1 of 4 adult mice inoculated intracerebrally with serum (blood drawn at 89 hours) from 1 of the 5 chickens developed mild convulsions on the 6th day following inoculation. Later this mouse was found dead, but brain tissue was not tested for the presence of virus. Serum obtained from the blood of the other 4 chickens failed to produce signs of encephalitis in mice.

TABLE I

Transmission of Virus

Suspension of infective mouse brain tissue → mosquito → chicken

Strain of virus	Source of virus for mosquitoes	Mosquitoes		Time after infective meal when transmission was attempted	No. of bites	Blood tested for virus	
		Species	Lot No.			By hen eggs to mice	By mice only
				days			
RN_6	Suspension of infective mouse brain tissue	*Culex quinquefasciatus*	C1-3	12	1	+	—
"	" "	*Anopheles punctipennis*	C4-1	8	1	+	—
"	" "	" *quadrimaculatus*	C3-1	9	2	+	?
"	" "	*Aëdes triseriatus*	C6-1	9	2	+	—
"	" "	" *aegypti*	C5-1	8	2	+	—

Viremia resulted in chickens from the bite of each of 5 species of mosquitoes which had ingested virus suspension 8 to 12 days previously and was demonstrated by chorioallantoic passage in the developing hen's egg (Table I).

Transmission of the Virus to Normal Chickens by Mosquitoes Infected by Feeding on Chickens Inoculated Subcutaneously

Seven lots of mosquitoes of 4 different species, (*Anopheles punctipennis* and *Culex pipiens*, 1 lot each; *Aëdes aegypti*, 2 lots; *Culex quinquefasciatus*, 3 lots), were allowed to feed on chickens inoculated subcutaneously with the Hubbard egg membrane strain. Viremia in these chickens was demonstrated by chorioallantoic inoculation before and after the mosquitoes had fed. Ten to 33 days after the infective meal each of the 7 lots of mosquitoes was given opportunity to feed on a normal chicken. Blood was drawn from 5 of these 7 chickens 36 to 50 hours after exposure to the mosquitoes. Each of the other 2 chickens was bled 3 times, on the 1st, 2nd, and 3rd days after exposure. Blood samples

were tested for virus by chorioallantoic passage. Viremia was demonstrated in all of the 7 chickens. Since transmission occurred in 2 instances in which no ingested blood was seen in the mosquitoes, it is possible that mere probing by the mosquito may be sufficient to transmit the virus in some instances.

The 7 successful transmissions to chickens were accomplished with mosquitoes utilized 10 to 33 days after the infective meal (Table II).

TABLE II

Transmission of Virus

Chicken inoculated subcutaneously → mosquito → chicken

Strain of virus	Source of virus for mosquitoes	Mosquitoes		Time after infective meal when transmission was attempted	No. of bites	Blood tested for virus	
	No. of inoculated chicken	Species	Lot No.			By hen eggs to mice	By mice only
				days			
Hubbard egg membrane	1–83	*Culex pipiens*	C19-2a	11–13	6	+	−
" " "	1–77	*Culex quinquefasciatus*	C10-2a	10–11	3	+	−
" " "	1–77	" "	C10-2b	33	1	+	−
" " "	1–83	" "	C10-3	13	?	+	−
" " "	1–65	*Anopheles punctipennis*	C11-1	10–11	?	+	−
" " "	1–65	*Aëdes aegypti*	C8-1	10–11	6	+	−
" " "	1–77	" "	C8-2	11–12	2	+	−

Transmission of the Virus to Normal Chickens by Mosquitoes Infected by Feeding on Chickens Infected by Mites

Transmission of virus by mosquitoes to normal chickens from chickens infected by mites was attempted 30 times using 27 different lots of mosquitoes. These lots included 5 species: *Anopheles punctipennis*, *Aëdes aegypti*, *Aëdes vexans*, *Culex quinquefasciatus*, and *Culex pipiens*. In the greater number of these experiments (20) *Culex pipiens* was used. In 17 of the 30 trials chickens bitten by mites of the Rippy colony constituted the source of virus for the mosquitoes. In the other 13 trials the chickens which served as the source of virus for mosquitoes were infected by the bite of mites experimentally infected with 1 of 3 strains of virus, the RN_6 strain, the Hubbard egg membrane strain, and the Mullen strain. In all chickens serving as a source of virus for mosquitoes, viremia was demonstrated during the period when the mosquitoes were fed. The periods allowed for incubation of virus in mosquitoes ranged from 5 to 55 days. One sample of blood was drawn from each of 27 chickens, 34 to 64 hours

TABLE III

Transmission of Virus

Mite → chicken → mosquito → chicken

Strain of virus infecting mites	Source of virus for mosquitoes	Mosquitoes		Time after infective meal when transmission was attempted	No. of bites	Blood tested for virus	
	No. of chicken with viremia owing to bite of mite	Species	Lot No.			By hen eggs to mice	By mice only
				days			
Rippy*	1–82	*Culex pipiens*	C18-3	10	11	+	−
"	1–84	" "	C18-4	15	1	+	−
"	1–94	" "	C18-5	16, 24	1, 1	+, +	−, 0
"	2–02	" "	C18-10	5	2	+	−
"	2–10	" "	C18-12	5, 12	1, 4	+, +	−, −
"	2–73	" "	C18-22	55	3	−	0
RN$_6$‡	1–85	" "	C24-2	15–16	6	+	−
"	1–85	" "	C24-1	16	1	+	−
"	2–11	" "	C24-6	18	20	+	−
"	2–64	" "	C24-12	54	4	−	0
Mullen‡	2–01	" "	C26-1	12	9	+	−
"	2–01	" "	C26-2	18	10	+	0
"	2–21	" "	C26-4	8, 27	35, 12	−, −	−, 0
"	2–23	" "	C26-5	10	10	+	0
Hubbard egg membrane‡	2–03	" "	C29-1	14	9	+	0
" " "	2–13	" "	C29-4	14	4	+	0
" " "	2–22	" "	C29-5	12	3	+	0
Rippy*	1–64	*Culex quinquefasciatus*	C14-1a	11	1	+	−
"	1–76	" "	C14-2	12	8	+	−
"	1–88	" "	C14-3	9	1	+	−
"	1–82	" "	C14-4	12	0?	−	−
RN$_6$‡	1–79	" "	C21-1	8–9	6	+	−
Rippy*	1–64	*Anopheles punctipennis*	C12-1	10	1	+	−
"	1–64	*Aëdes aegypti*	C7-1	10–11	3	−	−
"	1–76	" "	C7-2	12	1	+	−
"	1–94	" *vexans*	C25-1, 2	20	1	+	0
"	2–17	" "	C25-4	11	12	+	−

+ = virus isolated.

− = no virus isolated.

0 = blood not tested.

* Mites found infected in nature.

‡ Mites experimentally infected.

after exposure to infected mosquitoes. Each of the other 3 chickens was bled 3 times, on the 1st, 2nd, and 3rd days after exposure.

In this series of 30 experiments transmission of the virus to chickens by the bite of mosquitoes occurred 24 times (Table III). Chorioallantoic passage was necessary for the demonstration of viremia. Transmission was successful in at least one instance with each of the 5 species of mosquitoes used, and all 4 strains of virus were transmitted. In those experiments where transmission was successful, the period of incubation in the mosquito varied from 5 to 24 days. The 6 unsuccessful trials occurred with 5 lots of mosquitoes: 1 of *Aëdes aegypti*, 1 of *Culex quinquefasciatus*, and 3 of *Culex pipiens*. One lot of *Culex pipiens* failed to transmit the Mullen strain of virus at 8 days and again at 27 days following infective meal. The other 2 lots of *Culex pipiens* which gave negative results had been held for 54 to 55 days after the infective meal before they were tested for transmission of virus. In the one instance of *Culex quinquefasciatus* it was not certain whether any of the mosquitoes had attempted to bite the chicken. In the one instance where *Aëdes aegypti* gave negative results, mosquitoes were used for transmission test 10 to 11 days following the infective meal.

Control Experiments

Two types of controls were used. Mosquitoes bred in the laboratory were allowed to feed on normal chickens, hatched and bred in the laboratory, and after a 12 day interval were allowed to feed on other normal chickens. Blood samples drawn from the latter approximately 40 hours after exposure to the mosquitoes, were tested for virus by the chorioallantoic passage method. In the second type of control, mosquitoes were allowed a blood meal from chickens fed upon 2 to 3 days before by mites from a colony shown to be free of virus. After intervals of 12 to 14 days these mosquitoes were allowed a blood meal from normal chickens never exposed to mites. Two blood samples were drawn from each of the first group of chickens between 49 and 97 hours following exposure to mites and 1 blood sample was drawn from each of the second group of chickens at approximately 40 hours following exposure to mosquitoes. The blood samples were tested for virus, as before, by chorioallantoic passage. In none of the control chickens was there any evidence of viremia. Two control experiments of the first type were carried out, 1 with *Culex pipiens* and 1 with *Aëdes aegypti* and 3 of the second type, each with *Culex pipiens*.

In summary, the mosquitoes used in successful transmission experiments were infected with the St. Louis virus in 3 ways: (1) by ingesting a suspension of infected mouse brain tissue (RN_6 strain) in broth and defibrinated rabbit blood; (2) by feeding on chickens inoculated subcutaneously with the Hubbard egg membrane strain of virus; and (3) by feeding on chickens in which viremia resulted from the bite of naturally infected mites and mites experimentally infected with 3 strains of St. Louis virus. Control experiments of 2 types gave negative results consistently. Seven species of mosquitoes of 3 genera were used in these transmission experiments.

Transmission of the Virus to Young Mice by Infected Mosquitoes

In attempts to transmit the virus to mice, young Swiss mice, 6 to 14 days of age, were anesthetized lightly with dial and exposed to the bite of infected mosquitoes. Difficulties were encountered in that the young mice were not always able to withstand the procedure and in that mosquitoes did not bite mice readily. Satisfactory results were obtained in 9 instances only.

Of these 9 mice (10 to 14 days of age), 5 received only 1 bite each, 2 received 2 bites each, and 2 mice received 5 and 16 bites respectively. The 9 lots of mosquitoes included 1 of *Anopheles quadrimaculatus*, 1 of *Anopheles punctipennis*, 5 of *Aëdes aegypti*, 1 of *Aëdes triseriatus*, and 1 of *Culex quinquefasciatus*. The experiments with *Culex pipiens* were among those which were considered inconclusive. The source of virus for 4 of the 9 lots of mosquitoes was a suspension of infective mouse brain material. Two lots of the mosquitoes used were infected by feeding on a chicken inoculated with virus and 3 lots by feeding on a chicken bitten by infected mites. Periods ranging from 6 to 19 days were allowed for incubation of the virus in mosquitoes. None of the 9 mice developed signs of encephalitis: 3 were observed for a period of 21 days and then discarded; 4 were killed 10 days following the bite of infected mosquitoes, and the brain of each was passed intracerebrally to 4 adult mice; 2 of the 9 mice were killed 13 days following the bite of infected mosquitoes, and the brain of each was passed intracerebrally to 4 adult mice. No signs of encephalitis were noted in any of these passage mice. Four lots of the mosquitoes used in these experiments were infected by ingesting a a suspension of infective mouse brain tissue. Virus was isolated from triturated bodies of mosquitoes from 3 of these 4 lots. Nevertheless transmission of virus to mice by bite was not accomplished.

Transmission of the Virus to Hamsters by Infected Mosquitoes (Culex pipiens)

When difficulties were encountered in the transmission of virus to mice by infected mosquitoes, experiments with the Syrian hamster were undertaken. Since the hamster is large enough for bleeding at intervals, viremia tests were possible in addition to observation for signs of encephalitis. Two series of experiments were carried out (Table IV).

In the first series 13 young adult hamsters under dial anesthesia were exposed to mosquitoes which had fed on chickens bitten by infected mites. Viremia was demonstrated in all chickens used as a source of virus for mosquitoes. Transmission of the virus was attempted with 6 different lots of *Culex pipiens*, 8 to 24 days after ingestion of infective chicken blood. In 2 of these lots the infective meal was obtained from chickens bitten by mites of the Rippy colony, and in 4 lots the infective meal was obtained from chickens bitten by mites experimentally infected with the Mullen strain of virus. Four of the 13 hamsters succumbed immediately after the exposure period, apparently as a result of anesthesia; 2 of the 13 hamsters were observed for signs of encephalitis but were not tested for viremia; 6 were tested for viremia by bleeding twice at intervals between 40 and 90 hours, and 1 was bled 4 times at intervals between 40 and 108 hours after the beginning of exposure to mosquitoes. Of the 7 hamsters tested, 2 were shown to have viremia. In 1 hamster, which had been bitten by 9 mosquitoes 13 days after the infective meal (Rippy strain of virus), viremia was demonstrated at 64 hours but not at 40, 87, or 108 hours. In the other hamster bitten by 18 mosquitoes 8 days after the infective meal (Mullen strain of virus), viremia was demonstrated at 40 and at 68 hours after exposure. In each instance virus was isolated from heparinized blood by the chorio-

TABLE IV

Transmission of Virus

Mite → chicken → mosquito (*Culex pipiens*) → hamster

Series I

Strain of virus infecting mites	Source of virus for mosquitoes: No. of chicken with viremia owing to bite of mite	Mosquitoes (*Culex pipiens*) Lot No.	Time after infective meal when transmission was attempted	No. of bites	Viremia in hamsters (blood tested by hen eggs to mice): +	Viremia in hamsters (blood tested by hen eggs to mice): −	Remarks
			days		*hrs.*	*hrs.*	
Rippy*	1–94	C18-7	13	9	64	40, 87, 108	
"	2–02	C18-10‡	12	1		40, 90	
Mullen§	2–21	C26-3	8	18	40, 68		Died after second bleeding
"	2–21	C26-3	24	2		56, 80	
"	2–21	C26-4‖	19	25			Not bled
"	2–23	C26-6	17	2		40, 64	
"	2–23	C26-6	18	2		57, 79	Died 8 days after exposure to mosquitoes —virus not isolated from brain
"	2–23	C26-6	21	6		56, 80	
"	2–23	C26-5¶	18	5			Not bled

Series II

Rippy*	2–57	C18-15	8	8		50	
"	2–57	C18-15	11	3	48		0.03 ml. aleuronat intracerebrally
"	2–59	C18-18	12	1	26, 53, 73		
"	2–73	C18-22**	6	8	64		
"	2–73	C18-22**	15	15	56		" " " "
"	2–86	C18-24	16	2		57	" " " "
RN_6§	2–60	C24-9	4	25	50, 74	24	Died after third bleeding
"	2–60	C24-9	8	5		50	
"	2–60	C24-9	14	2	59		0.03 ml. aleuronat intracerebrally
"	2–60	C24-9	27	4	56		" " " "
"	2–60	C24-9	43	5		50	" " " "
"	2–64	C24-11	5	4	48		Died after bleeding
"	2–64	C24-12‡‡	8	1	26, 53		0.03 ml. aleuronat intracerebrally
"	2–87	C24-16	15	7		81	" " " "

* Mites found infected in nature.

‡ Transmitted virus to chicken 5 days after feeding.

§ Mites experimentally infected.

‖ Failed to transmit virus to chickens 8 and 27 days after feeding.

¶ Transmitted virus to chicken 10 days after feeding.

** Failed to transmit virus to chicken 55 days after feeding.

‡‡ Failed to transmit virus to chicken 54 days after feeding.

allantoic passage method. The hamster in which viremia was demonstrated at 40 and at 68 hours died as a result of the second bleeding on the 3rd day after exposure to mosquitoes. The second of the 2 hamsters shown to have viremia, the 5 in which viremia tests were negative, and the 2 which were not tested for viremia, a total of 8, did not develop signs of encephalitis. One of the hamsters which was tested for viremia with negative results, died 8 days following exposure to mosquitoes. Brain tissue from this hamster was passed intracerebrally to mice with negative results. The 7 hamsters which were observed for approximately 2½ months showed no signs of encephalitis.

In the second series of experiments, 17 young hamsters under dial anesthesia, were exposed to mosquitoes (*Culex pipiens*) 4 to 43 days after the mosquitoes had fed on chickens infected by mites. The colonies of mites used to infect the chickens serving as source of virus for the mosquitoes, were derived from mites found infected in nature (Rippy colony) and from mites experimentally infected with the RN_6 strain of virus. Virus was isolated by chorioallantoic passage from the blood of all chickens used as a source of virus for mosquitoes. Two of the 17 hamsters exposed to mosquitoes died before they had been bled for the viremia test, either as a result of anesthesia or of trauma produced by intracerebral injection of aleuronat. Of the remaining 15 hamsters, 1 was not tested for viremia; 1 or more blood samples from each of 14 were tested for virus by chorioallantoic passage at periods of 26 to 81 hours after the hamsters had been bitten by mosquitoes. In nine hamsters viremia was demonstrated at periods from 48 to 74 hours after exposure to mosquitoes; 2 of the 9 were bled 3 times, and viremia was demonstrated in 1 at 50 hours and at 74 hours but not at 24 hours, in the other at 26, 53, and 73 hours. A third hamster which was bled at 26 and at 53 hours showed viremia at both bleedings. Viremia was not demonstrated in 5 hamsters each of which was bled once at 50 to 81 hours after exposure to mosquitoes.

Of the 15 hamsters tested for viremia, 1 died immediately after bleeding; 14, which were observed for 2½ months showed no signs of encephalitis. With the idea of facilitating invasion of the brain by virus present in the blood, a number of hamsters were injected intracerebrally with aleuronat after exposure to infected mosquitoes. While 5 hamsters which had received aleuronat proved to have viremia, there was no apparent effect of the aleuronat in breaking the blood-brain barrier since not one of the 5 developed signs of encephalitis.

Several lots of mosquitoes were used more than once in the transmission experiments. In one instance mosquitoes which were used on 5 different occasions, transmitted the virus to hamsters 4, 14, and 27 days after the infective meal but not at 8 and 43 days after the infective meal. In another instance 1 lot of mosquitoes which transmitted the virus to a hamster at 11 days after the infective meal had not done so at 8 days after the infective meal. A third lot of mosquitoes transmitted the virus at 6 days and again at 15 days; in two other instances, 1 lot of mosquitoes which had transmitted the virus to a hamster 7 days after the infective meal, failed to transmit the virus to a chicken at 54 days, and another lot which had transmitted the virus to hamsters 6 and 15 days after the infective meal, failed to transmit the virus to a chicken at 55 days.

As Table IV shows virus was isolated in two series of experiments from the blood of 11 of 21 hamsters tested for viremia, demonstrating the transmission of virus by mosquitoes infected through feeding on chickens infected by mites. Eight lots of mosquitoes transmitted virus at periods varying from 4 to 27 days after the infective meal. Viremia was demonstrated on two occasions when one mosquito only was known to have bitten the hamster. In the other 9 instances in which viremia was demonstrated, the number of known bites varied from 2 to 25.

DISCUSSION

Within recent years evidence has been accumulating from field and laboratory studies which indicates that St. Louis encephalitis is an arthropod-borne disease. Isolation of the virus of St. Louis encephalitis from culicine mosquitoes collected in nature during epidemics and the transmission of the virus to experimental animals by the bite of mosquitoes emphasize the importance of this blood-sucking vector in the epidemiology of St. Louis encephalitis. The mosquito probably transmits the infection to higher animals and man. However, certain facts suggest that it is not the sole vector involved in the epidemiology of the disease. The virus has not been shown to persist in hibernating mosquitoes, nor has transfer of the virus in mosquitoes by way of the egg been demonstrated. While humoral antibodies to the virus of St. Louis encephalitis are present under natural conditions in vertebrates, particularly birds, there is no evidence that these animals constitute more than a transient source of virus. Apparently, virus remains in their blood for a few days only. Thus the question where the virus of this seasonal disease persists from year to year cannot be answered on the basis of the mosquito hypothesis alone. Also, there has been no adequate explanation of why epidemics occur rarely in certain localities although a few endemic cases occur there from year to year.

The isolation of the virus of St. Louis encephalitis from chicken mites (*Dermanyssus gallinae*) collected under natural conditions during non-epidemic years has pointed to the possibility that this arachnid vector might be a reservoir of the St. Louis virus. It has been shown that under laboratory conditions the virus is transferred through all stages of metamorphosis in the chicken mite, and that, once infected, a colony of chicken mites, by reason of transovarial passage, may remain infected for an indefinite period. By actual test it was shown that the virus remained in mites housed in the laboratory for a period of 3 years. While these findings suggest that *Dermanyssus gallinae* is serving as a natural reservoir, the possibility must be considered that this arachnid may be merely an accidental host, and hence of no epidemiologic significance. In order to determine whether the chicken mite is concerned in the natural transmission of St. Louis encephalitis, it is essential to know whether infected chicken mites feeding upon normal chickens can produce viremia. During the course of the present work this was accomplished many times, the blood of chickens fed upon by infected chicken mites being positive for virus for periods of 1 to 3 days, and in some instances 4 days, after the feeding period. It was demonstrated that mosquitoes feeding upon chickens infected by mites can acquire virus from the blood during the period of viremia, and that mosquitoes thus infected can transmit the virus to other chickens and to hamsters. Thus it is possible that in the epidemiology of St. Louis encephalitis two blood-sucking vectors may be involved—one an arachnid, the mite,

maintaining the virus by transovarial passage and the other, an insect, the mosquito, which carries the infection from birds to other vertebrates including man.

In these studies demonstration of viremia in animals fed upon by infected vectors presented technical difficulties since virus is present in small amounts in the blood of such animals. Passage on the chorioallantoic membrane was necessary in order to increase the virus to a level sufficient to produce signs of encephalitis in white Swiss mice. The question of multiplication of the virus in the body of the blood-sucking vectors was considered. In the case of the chicken mite, the demonstration of congenital transfer of the virus and the comparative ease with which virus could be isolated from mites of succeeding generations constitute indirect evidence that multiplication of the virus occurs. However, the present results give no convincing evidence that the virus multiplies in the body of the mosquitoes which were used in this investigation. On the other hand the amount of virus present in the body of the mosquitoes appeared to have a direct relation to the amount of virus ingested, even when a 2 week period of incubation was allowed; that is, virus was demonstrated readily by direct inoculation of mice, with extracts from mosquitoes which had fed on a suspension of brain tissue containing high concentration of virus, whereas extracts from mosquitoes which had fed on chickens infected by mites contained small amounts of virus, which could be demonstrated only by means of chorioallantoic passage.

Whether the virus undergoes changes in its characteristics, perhaps becoming less infective for vertebrates while maintaining itself in the body of an arthropod vector, is a question. The results reported here give no evidence of such a change in the mite since the virus from the bodies of chicken mites procured under natural conditions was infective for white mice without requiring passage for adaptation, and since uninfected chicken mites were infected successfully with laboratory-adapted strains: the Hubbard egg membrane strain and a strain isolated from the blood of a patient.[1] However, no detailed studies concerning this problem were undertaken.

While it was somewhat disappointing that the bite of infected mosquitoes which had acquired the virus from chickens infected by mites did not result in objective signs of encephalitis in hamsters or mice, viremia was demonstrated in hamsters in a significant number of instances. Even when efforts were made to break down the blood-brain barrier by the injection of aleuronat no signs of encephalitis were observed in these animals.

These observations suggest that the epidemiology of St. Louis encephalitis is a complex one, involving two blood-sucking vectors. A diagrammatic representation of this concept is given in Fig. 1. The chicken mite seems to be an

[1] Strain F 103, isolated from the mosquito in California, was sent to us through the courtesy of W. McD. Hammon. Several attempts to infect *Dermanyssus gallinae* with this strain have been made but up to the present time without success.

important reservoir vector in the St. Louis area. In other localities some other vector, probably an arachnid, may be playing a similar rôle; some species of mite, or a hard bodied tick, or a soft bodied tick are likely possibilities. Previous experiments of Blattner and Heys with the tick, *Dermacentor variabilis*, demonstrating that the virus of St. Louis encephalitis can be passed through the egg and into the next generation through the various stages of metamorphosis, suggested the potentiality of this arachnid as a reservoir for virus. However, in so far as we are aware, the St. Louis virus has not been encountered under natural conditions in any arachnid other than the chicken mite. The

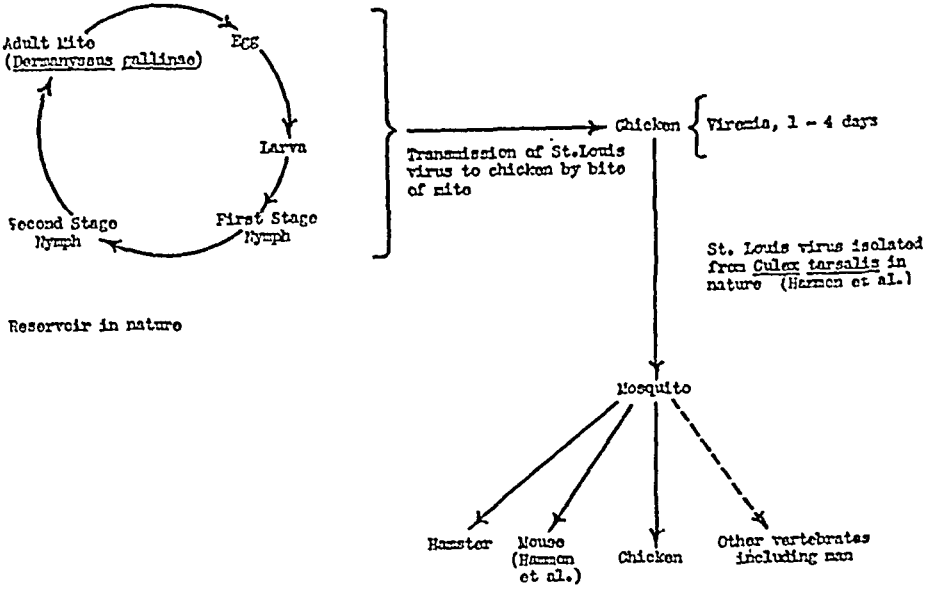

FIG. 1. The possible epidemiology of St. Louis encephalitis. ------ not yet proved; ——— proved experimentally.

concept of epidemiology outlined offers an explanation for the seasonal incidence of St. Louis encephalitis and for the persistence of the disease from year to year in a given community. Also it might explain adequately why so few major epidemics have been observed, since the requisite conditions appear to be exacting. Factors which probably would influence these conditions are temperature, relative humidity, the number of chickens in the community, the population of chicken mites, breeding places for mosquitoes, prevalence of certain species of mosquitoes, availability of susceptible vertebrates in the community, and the like. The few endemic cases which have been observed from year to year could be explained by assuming that in any given year relatively few mosquitoes might acquire the virus from chickens and that only a few individuals might be exposed to the bite of such infected mosquitoes.

Since the natural history of equine encephalomyelitis has many similarities to that of St. Louis encephalitis, it is conceivable that the same epidemiologic factors might be involved. The isolation by Sulkin (18) of the virus of equine encephalomyelitis from chicken mites collected in nature and the presence of the virus of equine encephalomyelitis in the bird mite, *Liponyssus sylviarum*, as shown by Reeves, Hammon, and their associates (19) lend support to this suggestion.

SUMMARY

The present experimental results concern primarily the question, whether or not mosquitoes feeding on chickens having viremia, as a result of the bite of infected mites, can acquire the virus of St. Louis encephalitis and whether or not mosquitoes thus infected, can transmit the virus to chickens and hamsters.

During the course of the investigation, 7 species of mosquitoes of 3 genera were infected with the virus in one or two or all of three ways: by feeding on a suspension of infected mouse brain tissue, by feeding on chickens in which viremia had been produced by subcutaneous inoculation of virus, and by feeding on chickens having viremia as a result of the bite of infected mites. These mosquitoes transmitted the virus to chickens at periods varying from 5 to 33 days after the infective meal.

The virus of St. Louis encephalitis was transmitted to hamsters by *Culex pipiens* at periods varying from 4 to 27 days after feeding on chickens having viremia as a result of the bite of infected mites. While viremia was demonstrated readily in hamsters, signs of encephalitis did not develop.

In all transmission experiments the method of chorioallantoic passage proved necessary for the demonstration of viremia.

A concept of the epidemiology of St. Louis encephalitis is presented: two blood-sucking vectors may be involved, one an arachnid, the mite, maintaining the virus in nature by transovarial passage, and the other, an insect, the mosquito, which carries the infection from birds to other vertebrates including man.

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THE CARCINOGENICITY OF CERTAIN DERIVATIVES OF *p*-DIMETHYLAMINOAZOBENZENE IN THE RAT*

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To determine how *p*-dimethylaminoazobenzene acts to produce liver tumors in the rat it is important to find what compound or set of compounds acts as the primary carcinogen (the compound(s) which directly initiates the carcinogenic process) when *p*-dimethylaminoazobenzene is the parent carcinogen (the compound administered). We have attacked this problem through investigations on the metabolism of this dye (1–4) and the effect of structure on its carcinogenic activity (5–7). The present paper reports further studies using the latter approach.

Previous studies on the aminoazo dyes indicated that at least one *N*-methyl group is probably necessary for strong carcinogenic activity. Thus Kinosita (8) found that *p*-diethylaminoazobenzene was inactive and Sugiura and his associates (9) have confirmed this observation. In addition the latter workers have shown that *p*-di-*n*-propylaminoazobenzene, *p*-di-*n*-butylaminoazobenzene, and *p*-di-*n*-amylaminoazobenzene are also inactive. Furthermore, the first metabolite of *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, has the same potency as the parent compound (6, 7, 9) while *p*-aminoazobenzene, the completely demethylated metabolite, is inactive under our conditions (6, 7). It is important to note that the first demethylation *in vivo* is reversible (4) while no reversal of the demethylation of *p*-monomethylaminoazobenzene has been detected (4, 6). The azo group, —N=N—, appears to be needed for activity since the reduction products of *p*-dimethylaminoazobenzene, N,N-dimethyl-*p*-phenylenediamine and aniline, are inactive (7–9); likewise the Schiff bases *p*-dimethylaminobenzalaniline and *p'*-dimethylaminobenzalaniline which contain the —CH=N— linkage are inactive (7). A report has appeared recently (10) on the carcinogenic activity of *p*-dimethylaminostilbene which has the —CH=CH— linkage; this compound appears to possess some carcinogenic activity towards the rat liver. The initial study (7) from this campus further demonstrated that the substitution of a methyl group for hydrogen in the positions *o*-(2-), *o'*-(2'-), *m'*-(3'-), or *p'*-(4'-) to the azo linkage produced wide variations in the activity of *p*-dimethylaminoazobenzene. This study

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resulted in the discovery of *m'*-methyl-*p*-dimethylaminoazobenzene as the most rapidly acting hepatic carcinogen and cirrhogen[1] known for the rat (5, 7, 11).

We have now extended these studies through (1) the determination of the carcinogenicity of the known and certain possible metabolites of *p*-dimethylaminoazobenzene singly and in various combinations and (2) further investigations on the carcinogenic activity of derivatives of *p*-dimethylaminoazobenzene obtained through substitution of the amino group or ring positions. A part of these data has been published in abstract form (3).

Preparation of Compounds

The compounds which have been tested for carcinogenicity in these experiments are listed in Tables I to III. In an earlier paper (7) the substituents on the rings of the azo dyes were designated as *o*-, *m*-, and *p*- and *o'*-, *m'*-, and *p'*- with respect to the azo linkage for substituents on the diamine and non-diamine rings, respectively. Since a few of the dyes reported here contain two substituents on one ring, the positions have been numbered by starting with the carbon attached to the azo linkage as 1 on the diamine ring and 1' on the non-diamine ring. Using this system *m'*-methyl-*p*-dimethylaminoazobenzene becomes 3'-methyl-4-dimethylaminoazobenzene. The number nomenclature is used in the remainder of this paper for the azo dyes.

The *p*-aminophenol, *o*-aminophenol hydrochloride, *p*-phenylenediamine, *p*-hydroquinone, and 4-aminoazobenzene as well as most of the intermediates used in the syntheses described below were obtained from the Eastman Kodak Co. The hydrochlorides of aniline, *m*-toluidine, *N*-methyl-*p*-phenylenediamine, and *N*, *N*-dimethyl-*p*-phenylenediamine were prepared from the redistilled amines by bubbling dry hydrogen chloride through a benzene solution of each compound. *N*-Methyl-*p*-phenylenediamine was obtained from *p*-nitro-*N*-methylaniline by reduction with stannous chloride. 2,4'-Diamino-5-dimethylaminodiphenyl was prepared by the reduction of 4-dimethylaminoazobenzene with stannous chloride (12); this amine was fed as the trihydrochloride. For the synthesis of 3-dimethylaminocarbazole[2], 3-nitrocarbazole (13) was reduced to the amine with sodium hydrosulfite. The amine was methylated with dimethyl sulfate in dilute ammonia solution and the crude product isolated as the methiodide. The free base (m.p. 114–116°) was obtained from the recrystallized methiodide by sublimation *in vacuo*. *N'*-Benzoyl-*N*, *N*-dimethyl-*p*-phenylenediamine (m.p. 223–225°) was prepared by the general Schotten-Baumann method.

All of the azo dyes except 4-aminoazobenzene were synthesized in this laboratory. The corrected melting point listed for each compound was obtained on the recrystallized product and, where the compound has been prepared previously, agrees with the value reported in the common chemical literature. The compounds which have not been described previously were purified by chromatography on alumina before being analyzed for total nitrogen; the determinations were made by the semi-micro Kjeldahl technique (14) after reduction with glucose.

4-Aminoazobenzene (m.p. 124.5–125.4°) was recrystallized twice from benzene-petroleum ether. 4-Formylaminoazobenzene (m.p. 162–162.5°) was synthesized through the reaction of formic acid with recrystallized 4-aminoazobenzene (15). Most of the *N*, *N*-dialkyl compounds were prepared by diazotizing 1 mole of the appropriate amine with 1 mole of sodium nitrite (concentrated water solution) in the presence of 2½ moles of hydrochloric acid in about 12 moles of water at 0° and adding the diazo solution to a 70 per cent ethanol solution of 1 mole of the

[1] *I.e.*, an agent causing cirrhosis.

[2] Dr. L. F. Small, National Institute of Health, Bethesda, Maryland, kindly gave us the details of this unpublished synthesis.

desired tertiary amine in the presence of 2 moles of sodium acetate at 10–15°. Except where noted the crude products were recrystallized from benzene-petroleum ether. The following compounds were prepared in this manner: 4-dimethylaminoazobenzene (m.p. 117–118°), 4-ethylmethylaminoazobenzene (m.p. 69.5–71.5°; per cent N, observed 17.50, 17.76, theoretical 17.52), 4-diethylaminoazobenzene (m.p. 96.5–98°), 4-benzylmethylaminoazobenzene (m.p. 99.5–100°; per cent N, observed 14.63, 14.73, theoretical 14.63), 4-β-hydroxyethylmethylaminoazobenzene (m.p., sinter 92°, melts 98–98.5°; per cent N, observed 16.59, 16.20, theoretical 16.46), 3′-methyl-4-dimethylaminoazobenzene (m.p. 119.5–120.5°), 2′, 4′-dimethyl-4-dimethylaminoazobenzene (m.p. 136–137°; per cent N, observed 16.57, 16.64, theoretical 16.59), 2′-nitro-4-dimethylaminoazobenzene (m.p. 125–126°), 3′-nitro-4-dimethylaminoazobenzene (m.p. 161–162°), 4′-nitro-4-dimethylaminoazobenzene (m.p. 227–229°), 2′-chloro-4-dimethylaminoazobenzene (m.p. 109–110°), 3′-chloro-4-dimethylaminoazobenzene (m.p. 103–104°), 4′-chloro-4-dimethylaminoazobenzene (m.p. 158–159°), and 3′-ethoxy-4-dimethylaminoazobenzene (m.p. 105–106°; per cent N, observed 15.58, 15.12, theoretical 15.60). 2′,5′-Dimethyl-4-dimethylaminoazobenzene (m.p. 157.5–158°; per cent N, observed 16.40, 16.56, theoretical 16.59) was prepared similarly except that it was necessary to filter the diazo solution prior to coupling to remove the unreacted amine hydrochloride. Coupling was carried out at 3° since the diazo solution was rather unstable at higher temperatures. In the synthesis of 3′, 5′-dimethyl-4-dimethylaminoazobenzene (m.p. 53–54°; per cent N, observed 16.24, 16.75, theoretical 16.59) the hydrochloride of 3,5-dimethylaniline (prepared from 3,5-dimethylnitrobenzene by reduction with stannous chloride) was so highly insoluble that the volume of the diazotization mixture was increased by using 3 moles of hydrochloric acid (and later 3½ moles of sodium acetate) and by adding a small amount of ethanol to make stirring of the crystalline mass possible. Since the crude 3′, 5′-dimethyl-4-dimethylaminoazobenzene did not solidify at 0°, the mixture was made alkaline and the residual amines were removed by steam distillation. The azo dye was then extracted with benzene and the solvent removed *in vacuo*. On cooling the residue a crystalline mass was obtained; it was further purified by adsorption on alumina from petroleum ether followed by elution with benzene and a final crystallization from petroleum ether at 0°.

For the preparation of the azo dyes containing only one alkyl substituent on the amino group equimolar amounts of benzenediazonium chloride (or a substituted benzenediazonium chloride) and the appropriate secondary amine were coupled in the presence of excess sodium acetate to yield the diazoamino compound. This intermediate was dried and then rearranged to the aminoazo dye by heating in the presence of 3 moles of the secondary amine and 0.7 mole of the hydrochloride of the secondary amine at 45–60° for 2 to 3 hours so that only a small evolution of nitrogen occurred. When the reaction was complete (dark red color) the azo dye was precipitated as the hydrochloride with excess 10 per cent hydrochloric acid. The crude hydrochloride was suspended in water and decomposed with excess alkali; the free base was recrystallized from benzene-petroleum ether. The yield of recrystallized product was about 40 per cent of theoretical. 4-Monomethylaminoazobenzene (m.p. 88.0–88.5°), 3′-methyl-4-monomethylaminoazobenzene (m.p. 109–110°; per cent N, observed 18.52, 18.33, theoretical 18.65), 4-monoethylaminoazobenzene (m.p. 86.5–87.5°; per cent N, observed 18.45, 18.43, theoretical 18.65) and 3-methyl-4-monomethylaminoazobenzene (m.p. 89–90°; per cent N, observed 18.54, 18.58, theoretical 18.65) were prepared in this manner. The *N*-methyl-*o*-toluidine used in the synthesis of the latter compound was prepared by converting redistilled *o*-toluidine to the *p*-toluenesulfonamide and methylating the recrystallized sulfonamide with dimethyl sulfate in alkaline solution. The sulfonamide of the *N*-methyl-*o*-toluidine was hydrolyzed by boiling in 20 per cent hydrochloric acid for 100 hours and the amine obtained by steam distillation of the hydrolysate after the addition of excess alkali.

The preparation of 3-methyl-4-dimethylaminoazobenzene has been hindered by the relative

inability of *o*-methyl-*N*, *N*-dimethylaniline to couple with benzenediazonium chloride (7). Attempts to methylate 3-methyl-4-monomethylaminoazobenzene with dimethyl sulfate in aqueous or methanolic alkali, with sodium and methyl iodide, or by a mixture of formic acid and formaldehyde were also unsuccessful. However, a few grams of the crude product were obtained in the following manner. One hundred and twenty-five ml. of a benzene solution containing 0.02 mole of 3-methyl-4-monomethylaminoazobenzene and 0.08 mole of dimethyl sulfate were kept at 37° for 2 days. The solvent was then removed *in vacuo*, and the residue was hydrolyzed at 70° for 1 hour in 100 ml. of 6 N potassium hydroxide in 25 per cent methanol. The mixture was diluted with an equal volume of water and extracted exhaustively with benzene. The volume of the extract was reduced *in vacuo*, 0.08 mole of dimethyl sulfate added, and the mixture again incubated as before. After isolating the dye in the manner described above, it was subjected to a third treatment with methyl sulfate in the same manner. The mixture of azo dyes extracted after the third methylation was refluxed for 2 to 3 hours with 0.15 mole of acetic anhydride in 15 ml. of pyridine. The acetylation mixture was then diluted to 75 ml. with benzene, and the 3-methyl-4-dimethylaminoazobenzene was removed by 4 extractions with 30 ml. portions of 5 N hydrochloric acid. The *N*-acetyl-3-methyl-4-monomethylaminoazobenzene was not extracted under these conditions. After the addition of excess alkali to the acid solution, the 3-methyl-4-dimethylaminoazobenzene was extracted with benzene and the product was obtained by removal of the solvent *in vacuo*. The over-all yield from the monomethyl dye was 30 to 40 per cent. The dimethyl dye did not crystallize and, due to the limited supply, was fed as such. When analyzed by chromatography on an alumina column (16) only one major homogeneous band and traces of the starting material were found. The chromatographically pure band yielded an oil which could not be crystallized but which contained 17.11, 17.38 per cent nitrogen (theoretical 17.52). We have already discussed (7) the note of Hantzsch who reported a melting point but not his method of synthesis for this compound.

4-Hydroxyazobenzene (m.p. 155–156°) was prepared by coupling equimolar quantities of benzenediazonium chloride and phenol in alkaline solution at 10–15°. For the synthesis of 4′-hydroxy-4-aminoazobenzene (m.p. 180–181° decomposes) *N*-acetyl-*p*-phenylenediamine was diazotized in the usual manner and similarly coupled with phenol. The mixture was then neutralized to pH 7, the crude *N*-acetyl-4′-hydroxy-4-aminoazobenzene filtered off, and finally hydrolyzed under reflux with excess 5 per cent sodium hydroxide. 4′-Hydroxy-4-monomethylaminoazobenzene (m.p. 196–198° decomposes; per cent N, observed 18.28, 18.16, theoretical 18.49) was obtained from *N*, *N*-acetylmethyl-*p*-phenylenediamine and phenol in the same manner. The diamine was prepared by the reduction of *p*-nitro-*N*-acetylmethylaniline with stannous chloride. In the preparation of 4′-hydroxy-4-dimethylaminoazobenzene (m.p. 201–202° decomposes) *p*-aminophenol was diazotized and coupled with dimethylaniline; coupling did not take place immediately but after 1 to 2 days at 0–5° a crystalline mass precipitated.

Since the diazotization of *o*-aminophenol yields a diazo oxide which will not couple with dimethylaniline, 2′-hydroxy-4-dimethylaminoazobenzene (m.p. 143–144°; per cent N, observed 17.60, 17.32, theoretical 17.42)[3] was prepared as follows. *o*-Nitrophenol was esterified with *p*-toluenesulfonyl chloride in pyridine solution at 45°. The nitro compound was reduced to the *p*-toluenesulfonyl ester of *o*-aminophenol by adding gradually an aqueous solution of 3 moles of sodium hydrosulfite to an ethanol solution of 1 mole of the nitro ester at 60–80°. The resulting amine was then diazotized and coupled with dimethylaniline by the usual procedure. After saponification of the azo ester under reflux for 2 hours in ethanolic sodium hydroxide the 2′-hydroxy-4-dimethylaminoazobenzene precipitated upon neutralization. 3′-Hydroxy-4-

[3] We are indebted to Mrs. Virginia M. Kline for assistance in the syntheses of these hydroxyaminoazo dyes.

dimethylaminoazobenzene (m.p. 139.5–140°; per cent N, observed 17.54, 17.23, theoretical 17.42) was prepared in the same manner to prevent the coupling of the diazotized *m*-aminophenol with itself. The *p*-toluenesulfonyl ester of *m*-nitrophenol was prepared as described above; the reduction was also similar to that already described except that a mixture of 3 per cent acetone and 97 per cent ethanol was needed to dissolve the nitro ester. Due to its insolubility it was necessary to diazotize the *p*-toluenesulfonyl ester of *m*-aminophenol as a fine suspension in a 60 per cent ethanol solution of hydrochloric acid. Coupling of the diazonium compound and saponification of the azo ester followed the procedures described above. All of the hydroxyaminoazo dyes were recrystallized from ethanol-water.

Method of Assay

With the exceptions to follow each compound listed in Tables I to III was fed to 12 young adult male albino rats of the Sprague-Dawley strain and 150 to 200 gm. in weight. Because of a limited supply of the compounds only 10 rats were fed 3-dimethylaminocarbazole, and 6 rats each the 2,4′-diamino-5-dimethylaminodiphenyl and 3-methyl-4-dimethylaminoazobenzene. Each of the monophenyl amines and *p*-hydroquinone was fed to groups of 6 rats. Our previous experience with the latter compounds in metabolic tests indicated that they possessed little toxicity when fed at such levels and would probably prove to be non-carcinogenic. Six to 16 compounds were tested simultaneously, and each series was controlled by a group of 12 rats fed 0.06 per cent of 4-dimethylaminoazobenzene. All of the rats were fed *ad libitum* a semisynthetic diet (7) consisting of crude casein, 120 gm.; corn oil, 50 gm.; rice-bran concentrate (vitab), 20 gm.; salt mixture, 40 gm.; and glucose monohydrate, 770 gm. The hydroxyaminoazo dyes were added to the diets in a small volume of acetone, while the monophenylamines, *p*-hydroquinone, 3-dimethylaminocarbazole, and 2,4′-diamino-5-dimethylaminodiphenyl were ground with a few grams of glucose before being added to the diets. All of the remaining compounds were dissolved with heat in the corn oil of the diets. One drop of halibut liver oil was fed monthly, and the animals were housed in groups of 6 in screen-bottomed cages. In general, at the end of the dye-feeding periods indicated in the tables the livers were examined by laparotomy and the rats were then fed the same diet without the test compound for an additional 2 months (17). In the case of the monophenylamines (Table I, groups 12 to 16; Table II, groups 10 to 12) the rats were fed the compounds continuously for 9 months and then killed.

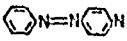
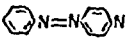
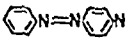
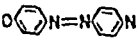
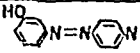
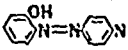
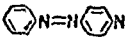
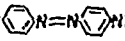
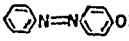
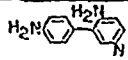
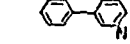
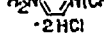
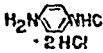
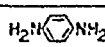
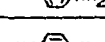
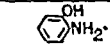
RESULTS

The results of the tests on the carcinogenicity of the compounds related to 4-dimethylaminoazobenzene are listed in Tables I to III. The three control groups fed 4-dimethylaminoazobenzene had tumor incidences of 70 to 92 per cent at 4 months and 100 per cent at 6 months. However, since some variation is observed in groups fed at various times, the control group selected for each table was the group fed 4-dimethylaminoazobenzene simultaneously with the most active compounds included in that table. All of the azo dyes, except 4-aminoazobenzene, were fed at molar levels equivalent to 0.06 per cent of 4-dimethylaminoazobenzene, but the times of feeding varied with the activity of the compound. Accordingly, the following formula has been used to obtain a *rough* index of relative activity of these compounds:

$$\text{Relative activity} = \frac{6 \times 4 \times \text{per cent tumors with test compound}}{\text{Months fed} \times \text{per cent tumors with 4-dimethylaminoazobenzene}}$$

TABLE I

The Carcinogenicity of Various Known and Possible Metabolites of 4-Dimethylaminoazobenzene

GROUP	COMPOUND FED *	PRODUCT OF METABOLISM IN THE RAT	LEVEL IN THE DIET		TIME COMPOUND WAS FED	GROSS CIRRHOSIS AT END OF FEEDING COMPOUND	TUMOR INCIDENCE PER CENT		
			PERCENT	MILLI-MOLS / KGM.	MOS.		4 MOS.	6 MOS.	9 MOS.
1	 4-DIMETHYLAMINOAZOBENZENE	+	.060	2.67	4	MOD.-SEVERE	80	100	—
2	 4-MONOMETHYLAMINOAZOBENZENE	+	.056	"	"	"	85	100	—
3	 4-AMINOAZOBENZENE	+	.106	5.34	9	NONE	0	0	0
4	 4'-HYDROXY-4-DIMETHYLAMINOAZOBENZENE	?	.064	267	6	"	0	0	0
5	 3'-HYDROXY-4-DIMETHYLAMINOAZOBENZENE	?	"	"	"	"	0	0	0
6	 2'-HYDROXY-4-DIMETHYLAMINOAZOBENZENE	?	"	"	"	"	0	0	0
7	 4'-HYDROXY-4-MONOMETHYLAMINOAZOBENZENE	+	.061	"	"	"	0	0	0
8	 4'-HYDROXY-4-AMINOAZOBENZENE	+	.057	"	"	"	0	0	0
9	 4-HYDROXYAZOBENZENE	?	.053	"	"	"	0	0	0
10	 2,4'-DIAMINO-5-DIMETHYLAMINODIPHENYL	?	.176	5.34	4	"	0	0	0
11	 3-DIMETHYLAMINOCARBAZOLE	?	.056	2.67	"	"	0	0	0
12	N,N-DIMETHYL-p-PHENYLENE DIAMINE DIHYDROCHLORIDE + ANILINE HYDROCHLORIDE 	?	.168	8.01	9	"	0	0	0
		+	.105	"					
13	N-METHYL-p-PHENYLENE DIAMINE DIHYDROCHLORIDE + ANILINE HYDROCHLORIDE 	+	.156	"	"	"	0	0	0
		+	.105	"					
14	p-PHENYLENE DIAMINE + ANILINE HYDROCHLORIDE 	+	.084	"	"	"	0	0	0
		+	.105	"					
15	p-AMINOPHENOL 	+	.087	"	"	"	0	0	0
16	o-AMINOPHENOL HYDROCHLORIDE 	+	.117	"	"	"	0	0	0

* SEE ALSO GROUPS 8, 11, AND 12 - TABLE II

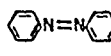
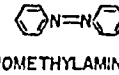
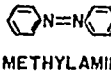
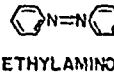
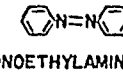
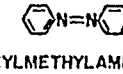
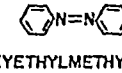
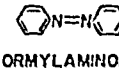
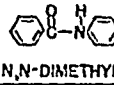
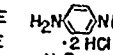
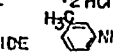
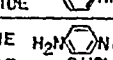
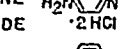
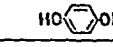
where the activity of 4-dimethylaminoazobenzene has been designated arbitrarily as 6 when this dye is fed for 4 months. The per cent of tumors at the end of the dye-feeding was generally used for calculation; however, if no tumors had appeared at this time the incidences at the end of the experiment were substituted. In general, the extent of the cirrhosis and, except where noted, the toxicity (weight loss) of the various compounds paralleled their carcinogenic activities. The survival of the rats fed the azo dyes ranged from 83 to 100 per cent at the end of the dye-feeding period and 100 per cent survival was obtained with all of the other compounds.

Table I lists the compounds which are either known to be metabolites of 4-dimethylaminoazobenzene or which have been suggested as possible derivatives *in vivo*. As reported earlier (6) 4-monomethylaminoazobenzene (group 2) has an activity equal to that of the dimethyl compound; none of the other compounds showed any activity either as a carcinogen or as a cirrhogen under these conditions. 4-Aminoazobenzene was inactive when fed at twice the molar level for 9 months. Similarly 4′-hydroxy-4-aminoazobenzene, 4′-hydroxy-4-monomethylaminoazobenzene, 4-hydroxyazobenzene, and 4′-hydroxy-, 3′- hydroxy-, and 2′-hydroxy-4-dimethylaminoazobenzene were all inactive when fed at molar levels equivalent to 4-dimethylaminoazobenzene for 6 months. However, 4′-hydroxy-4-dimethylaminoazobenzene caused about twice as great a loss in weight as 4-dimethylaminoazobenzene while the other non-carcinogenic azo dyes were much less toxic in this respect than the reference compound. 2,4′-Diamino-5-dimethylaminodiphenyl and 3-dimethylaminocarbazole were also inactive when fed for 4 months. *p*-Phenylenediamine and its *N*-methyl and *N*,*N*-dimethyl derivatives were non-carcinogenic when fed at 3 times the molar level for 9 months. *N*,*N*-Dimethyl-*p*-phenylenediamine was also non-carcinogenic when fed at 3 times the molar level with *m*-toluidine or *p*-hydroquinone, which might act as an antioxidant to preserve the diamine in the diet and digestive tract (Table II, groups 10 and 11); no activity was observed when the diamine was fed as the benzamide (Table II, group 9). *o*-Aminophenol and *p*-aminophenol were likewise inactive (Table I). Finally, neither cirrhosis nor tumors were obtained when a mixture of the following known and possible metabolites was fed to 15 rats for 11 months: 0.04 per cent of 4-aminoazobenzene and 0.01 per cent (calculated as free base) each of 4′-hydroxy-4-dimethylaminoazobenzene, 4′-hydroxy-4-monomethylaminoazobenzene, 4′-hydroxy-4-aminoazobenzene, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride *N*-monomethyl-*p*-phenylenediamine dihydrochloride, *p*-phenylenediamine, aniline hydrochloride, and *p*-aminophenol.

The type of alkyl group combined with the amino group has a marked effect on the carcinogenicity of the aminoazo dyes (Table II). At least one methyl group appears to be essential for activity; thus 4-diethylaminoazobenzene and 4-formylaminoazobenzene were inactive while 4-dimethyl-, 4-monomethyl-,and

TABLE II

The Carcinogenicity of Certain N-Substituted Derivatives of 4-Aminoazobenzene

GROUP	COMPOUND FED	PER CENT IN DIET	TIME COMPOUND WAS FED MOS.	GROSS CIRRHOSIS AT END OF FEEDING COMPOUND	TUMOR INCIDENCE PER CENT 4 MOS.	6 MOS.	8 MOS.	RELATIVE ACTIVITY OF COMPOUND
DYES WITH VARIOUS SUBSTITUENTS ON THE AMINO GROUP (LEVEL IN DIET = 2.67 MILLIMOLS / KGM.)								
1	 4-DIMETHYLAMINOAZOBENZENE	.060	4	MOD.-SEVERE	92	100	—	6 REF. COMP
2	 4-MONOMETHYLAMINOAZOBENZENE	.056	"	"	85	100	—	6
3	 4-ETHYLMETHYLAMINOAZOBENZENE	.064	"	"	84	100	—	6
4	 4-DIETHYLAMINOAZOBENZENE	.067	6	NONE	0	0	0	0
5	 4-MONOETHYLAMINOAZOBENZENE	.060	"	"	0	0	0	0
6	 4-BENZYLMETHYLAMINOAZOBENZENE	.077	"	"	0	0	0	0
7	 4-β-HYDROXYETHYLMETHYLAMINOAZOBENZENE	.068	"	"	0	0	0	0
8	 4-FORMYLAMINOAZOBENZENE	.060	"	"	0	0	0	0
MISCELLANEOUS COMPOUNDS								
9	 N'-BENZOYL-N,N-DIMETHYL-p-PHENYLENE DIAMINE	.064	6	"	0	0	0	0
10	N,N-DIMETHYL-p-PHENYLENE DIAMINE DIHYDROCHLORIDE  ·2 HCl m-TOLUIDINE HYDROCHLORIDE  HCl	.168 .117	9	"	0	0	0 (9 MOS.)	0
11	N,N-DIMETHYL-p-PHENYLENE DIAMINE DIHYDROCHLORIDE  ·2 HCl p-HYDROQUINONE 	.168 .029	"	"	0	0	"	0
12	p-HYDROQUINONE 	.029	"	"	0	0	"	0

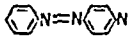
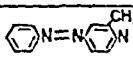
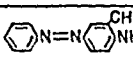
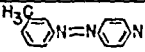
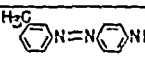
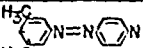
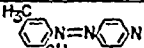
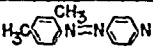
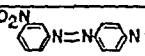
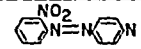
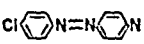
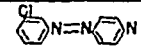
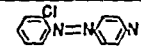
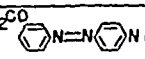
4-ethylmethyl aminoazobenzene were equally carcinogenic. However, the presence of one *N*-methyl group is not sufficient to insure carcinogenicity since 4-benzylmethylaminoazobenzene and 4-β-hydroxyethylmethylaminoazobenzene were inactive.

In an effort to rationalize the different activities of the aminoazo dyes with at least one methyl group on the amino nitrogen, the blood and livers of rats fed these compounds were analyzed for aminoazo dyes as described previously (16). It was found possible to separate mixtures of aminoazo dyes into groups of primary, secondary, and tertiary compounds on an aluminum oxide column, but mixtures of two or more secondary or tertiary aminoazo dyes could not be resolved sufficiently in this manner to make work with microgram quantities practical. Accordingly, the azo dyes present in the livers could only be analyzed qualitatively for the presence or absence of each class of compounds. Since 4-aminoazobenzene was found to be the only azo dye present in the blood when any of these dyes were fed, its level could be determined quantitatively. The livers of rats fed 4-dimethyl-, 4-monomethyl-, 4-ethylmethyl-, or 4-diethylaminoazobenzene contained all three classes of azo dyes while the livers from rats fed 4-monoethylaminoazobenzene contained primary and secondary aminoazo dyes with possible traces of tertiary compounds. Similar data have been obtained by others for the diethyl compound (18). In all of these cases the primary aminoazo dye (4-aminoazobenzene) accounted for more than half of the total azo dye present. However, when 4-β-hydroxyethylmethylaminoazobenzene or 4-benzylmethylaminoazobenzene was fed, the aminoazo dye was found to be largely tertiary with only traces of the primary and secondary compounds. The analyses for 4-aminoazobenzene in the blood were in agreement with these observations since 12 to 15 μg. were found per ml. of blood from rats fed 4-dimethyl-, 4-monomethyl-, 4-diethyl-, 4-monoethyl, or 4-ethylmethylaminoazobenzene while only 1 to 2 μg. per ml. were present in the blood from rats fed 4-benzylmethyl- or 4-β-hydroxyethylmethylaminoazobenzene. 4-Di-β-hydroxyethylaminoazobenzene also gives rise to only low levels of 4-aminoazobenzene in the blood (19). Thus it would appear that the rat can demethylate or deethylate certain aminoazo dyes quite readily, but removes benzyl or β-hydroxyethyl groups from such dyes slowly. Further, since 4-ethylmethylaminoazobenzene is strongly carcinogenic while 4-diethylaminoazobenzene and 4-monoethylaminoazobenzene are inactive, it seems probable that the ethyl group may be removed prior to the methyl group from the ethylmethyl derivative and thus yield the active 4-monomethylaminoazobenzene *in vivo*.

Further experiments on the effect of ring substituents on the potency of 4-dimethylaminoazobenzene are presented in Table III. In agreement with other studies (5, 7, 11) 3′-methyl-4-dimethylaminoazobenzene was much more active than the reference compound, and its monomethyl derivative, 3′-methyl-4-monomethylaminoazobenzene, was found to be as active as the dimethyl

TABLE III

The Carcinogenicity of Various Ring-Substituted Derivatives of 4-Dimethylaminoazobenzene

GROUP	COMPOUND FED	PER CENT IN DIET	TIME COMPOUND WAS FED MOS.	GROSS CIRRHOSIS AT END OF FEEDING COMPOUND	TUMOR INCIDENCE PER CENT 3 MOS.	4 MOS.	7 MOS.	9 MOS	RELATIVE ACTIVITY OF COMPOUND
1	 4-DIMETHYLAMINOAZOBENZENE	.060	4	MOD.-SEVERE	0	70	100	–	6 REF. CO
2	 3-METHYL-4-DIMETHYLAMINOAZOBENZENE	.064	7	NONE	–	0	0	0	0
3	 3-METHYL-4-MONOMETHYLAMINOAZOBENZENE	.060	11	NONE-MILD	–	0	0	9	<1
4	 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE	.064	3	MOD.-SEVERE	30	88	–	–	10-12 SEE TE
5	 3′-METHYL-4-MONOMETHYLAMINOAZOBENZENE	.060	11	11	33	92	–	–	10-12 SEE TE
6	 3,5′-DIMETHYL-4-DIMETHYLAMINOAZOBENZENE	.067	7	NONE-MILD	–	0	0	0	0
7	 2,5′-DIMETHYL-4-DIMETHYLAMINOAZOBENZENE	11	11	NONE	–	0	0	0	0
8	 2,4′-DIMETHYL-4-DIMETHYLAMINOAZOBENZENE	11	11	11	–	0	0	0	0
9	 3′-NITRO-4-DIMETHYLAMINOAZOBENZENE	.072	4	MILD-MOD.	–	70	83	–	5
10	 2′-NITRO-4-DIMETHYLAMINOAZOBENZENE	11	11	NONE-MILD	–	0	46	–	3
11	 4′-CHLORO-4-DIMETHYLAMINOAZOBENZENE	.068	5	MILD-MOD.	–	0	27	–	1-2
12	 3′-CHLORO-4-DIMETHYLAMINOAZOBENZENE	11	4	MOD.	–	70	92	–	5-6
13	 2′-CHLORO-4-DIMETHYLAMINOAZOBENZENE	11	11	MILD-MOD.	–	23	31	–	2
14	 3′-ETHOXY-4-DIMETHYLAMINOAZOBENZENE	.072	7	NONE	–	0	9	18	<1

compound. When 3′-methyl-4-dimethylaminoazobenzene and 4-dimethylaminoazobenzene have been compared at various levels of dye and for various times of feeding, the 3′-methyl derivative has been found to be approximately twice as active as the reference compound (11) hence it has been given a relative activity of 10 to 12 even though the present experiments were not designed to measure such strong activity. 3-Methyl-4-monomethylaminoazobenzene induced a liver tumor in 1 of 12 rats at 9 months and was assigned an activity of less than 1. 3-Methyl-4-dimethylaminoazobenzene did not produce any tumors in 6 rats; however, the preparation was non-crystalline and only a small number of animals were used, so that it is quite probable that the 2 compounds would be equally active (though very weak) under appropriate conditions. The derivatives of 4-dimethylaminoazobenzene containing ring methyl groups in the 3′ and 5′, 2′ and 5′, and the 2′ and 4′ positions were all inactive when fed for 7 months. 3′-Ethoxy-4-dimethylaminoazobenzene had a potency of less than 1. Introduction of a chloro group in the 3′ position gave a compound about as active as 4-dimethylaminoazobenzene while the 2′-chloro and 4′-chloro derivatives were one-third and one-fourth as active, respectively. Similarly, 3′-nitro-4-dimethylaminoazobenzene was about five-sixths as carcinogenic as the reference compound and 2′-nitro-4-dimethylaminoazobenzene was only half as active. It was also planned to include 4′-nitro-4-dimethylaminoazobenzene in this series, but its high melting point (229°) and low solubility in oil suggested that it might not be absorbed readily from the tract. When qualitative tests on the feces of rats fed the dye indicated that large amounts were excreted unchanged, the carcinogenicity test was discontinued.

At the completion of all of the tests for carcinogenic activity, pairs of rats were fed each of the azo dyes (at a level equivalent to 0.06 per cent of 4-dimethylaminoazobenzene) included in this report as well as the various C-monomethyl derivatives of 4-dimethylaminoazobenzene which were studied previously (7). Feces from each rat were tested qualitatively for aminoazo dyes by crushing in 7 N HCl (and 0.5 N HCl for the hydroxyaminoazo dyes); less than 100 μg. of any of these dyes gives an intense red color under these conditions. In those cases where the qualitative tests were positive, quantitative collections of the feces were made for two successive 24 hour periods and the dye was removed from the dried feces by exhaustive extraction with acetone in a Soxhlet apparatus. Approximately 2 per cent of the administered 4′-chloro-4-dimethylaminoazobenzene, 15 per cent of the 4′-methyl-4-dimethylaminoazobenzene, 40 per cent of the 3′-nitro-4-dimethylaminoazobenzene, and 45 per cent of the 4′-nitro-4-dimethylaminoazobenzene were excreted in the feces. Rats fed 4-dimethylaminoazobenzene excreted less than 0.1 per cent of the administered dye by this route (4) and the rats fed the remaining dyes excreted less than 1 per cent. These data indicate that the real carcinogenic activities of 4′-methyl-and 3′-nitro-4-dimethylaminoazobenzene are probably greater by at

least 20 and 60 per cent, respectively, than the values obtained. As a result of these findings 4′-nitro-4-dimethylaminoazobenzene is again under test.

DISCUSSION

It is of considerable interest that of the 18 known or possible metabolites of 4-dimethylaminoazobenzene tested in these experiments only one, 4-monomethylaminoazobenzene, has exhibited carcinogenic activity. This compound possesses the same carcinogenicity as its precursor (6) and, further, since each of these dyes is converted into the other *in vivo* (4), they can best be described as metabolites of each other. However, 4-aminoazobenzene, another known metabolite (4), has proven inactive even when fed twice as long at twice the molar level as the methylated dyes. This observation, which is in agreement with previous work (6, 7, 9, 20) parallels our failure to detect *in vivo* any methylation of 4-aminoazobenzene to the monomethyl dye (6). The work of Kirby (21) indicating that 4-aminoazobenzene may have carcinogenic activity of a very low order has been discussed elsewhere (6). Although evidence exists for the formation of the 4′-hydroxy derivatives of 4-monomethylaminoazobenzene and 4-aminoazobenzene in the rat following the ingestion of either the former dye or its dimethylamino precursor (2, 3) neither of these hydroxy dyes was active. Likewise, the 2′-, 3′-, and 4′-hydroxy derivatives of 4-dimethylaminoazobenzene, the possible product of deamination, 4-hydroxyazobenzene, and a possible metabolite of 4-monomethylaminoazobenzene, 4-formylaminoazobenzene, all proved inactive. It is to be noted that the benzidine rearrangement product of 4-dimethylaminoazobenzene, 2,4′-diamino-5-dimethylaminodiphenyl, was inactive even when fed at twice the molar level as its supposed precursor, 4-dimethylaminoazobenzene. No valid evidence has been adduced that this compound is produced *in vivo* from 4-dimethylaminoazobenzene although the results of the color test of Elson and Warren (22) applied to the urine of rats fed the dye have been construed as such (22, 23). This diphenyl derivative, like the simpler diamines theoretically derivable from the parent dye, has been the subject of an undue amount of speculation (22-24); the actual test for the carcinogenicity of this compound is recorded here for the first time. We also tested the activity of 3-dimethylaminocarbazole and found it to be zero. This compound was fed since it could conceivably be formed by a deaminative cyclization of a rearrangement product of the hypothetical metabolite, 4-dimethylaminohydrazobenzene. A similar set of reactions have been suggested by Cook and his coworkers (25) for the metabolism of the hepatic carcinogen, 2,2′-azonaphthalene, in the mouse. In that species the two proposed metabolites of the hypothetical intermediate, 2,2′-hydrazonaphthalene: 2,2′-diamino-1,1′-dinaphthyl and 3, 4, 5, 6,- dibenzcarbazole, were each found to be carcinogenic for the mouse liver. Nor did any of the mixtures of amines derivable by complete reduction of the azo linkage in the highly active

carcinogens 4-dimethylaminoazobenzene, 4-monomethylaminoazobenzene, or 3'-methyl-4-dimethylaminoazobenzene prove to have any activity. The known metabolites, *p*-phenylenediamine (2, 3, 26), *p*-aminophenol (2, 3, 26), and *o*-aminophenol (2, 3) also were inactive. Finally, a mixture of 9 possible and known metabolites of 4-dimethyl- and 4-monomethylaminoazobenzene proved inactive when fed for 11 months. This mixture was fed before *o*-aminophenol was found to be a metabolite of the dimethyl dye. Recently White and her associates (27) have found a very low incidence of liver tumors in rats fed aniline for 15 to 31 months. As indicated below the low activity of this metabolite may have no significance in carcinogenesis by 4-dimethylaminoazobenzene.

Thus of the possible and known metabolites of 4-dimethylaminoazobenzene that have been tested 4-monomethylaminoazobenzene at this time stands alone in its ability to act as a hepatic carcinogen. This led us to the tentative conclusion (3) that in the rat 4-dimethylaminoazobenzene itself, 4-monomethylaminoazobenzene, a mixture of these dyes, or an azo dye closely related to these dyes is the primary carcinogen when 4-dimethylaminoazobenzene is fed. However, it is important to note that activity, positive or negative, of a possible or proven metabolite *cannot necessarily* either implicate or eliminate it as responsible for the activity of the parent compound. Thus, while the testing of metabolites is a necessary first step it alone cannot supply conclusive evidence; supporting data of a more direct nature are necessary. We feel that we have partially supplied this in our recent work (1) on the protein-bound dyes present in the livers of rats fed 4-dimethylaminoazobenzene. Our present position on the question of how 4-dimethylaminoazobenzene acts to produce liver tumors in the rat is based on the several correlations found between the level of the protein-bound dyes in the liver and the probability that the liver will later develop a tumor (1) and the data given in the present and other papers (6, 7). Thus we suggest that the primary carcinogen consists of either or both of the protein-bound dyes, 4-monomethylaminoazobenzene and an unidentified polar aminoazo dye, and that the formation of bound dye constitutes one of the first steps in the carcinogenic process induced by the parent dye (1).

Many of the compounds listed in Tables I to III were also tested with the view of increasing the information available on the structural features necessary for strong carcinogenic activity of aminoazo dyes and related compounds. Approximately 40 aminoazo dyes and amines were tested and only a few of the compounds, all azo dyes, were carcinogenic under our conditions. Alterations in the azo linkage, in the substituents of the amino group, and in the ring substituents have been tested. In our previous work the replacement of the azo linkage in 4-dimethylaminoazobenzene by the easily hydrolyzed —N=CH— or —CH=N— linkages produced inactive compounds (7); in the present experiments the substitution of the amide linkage, —CO—NH—, as in the benzamide of dimethyl-*p*-phenylenediamine, also led to an inactive

compound. However, the substitution of an ethylene linkage for the azo group as in 4-dimethylaminostilbene produced a compound with a greatly lowered carcinogenic activity for the rat liver (10). When the methyl groups in the parent dye were replaced by 2 H—, H— and CH_3CH_2—, or 2 CH_3CH_2— groups, compounds of zero activity resulted. However, if the amino group carried H— and CH_3— or CH_3— and CH_3CH_2— the activities of these dyes were equal to that of 4-dimethylaminoazobenzene. In contrast the dyes with the amino group substituents CH_3— and $HOCH_2CH_2$—, CH_3— and phenyl—CH_2— or H— and CHO— were inactive. Substituents in the rings also produced great variations in activity. Calculations of the relative activities of the ring-monomethyl derivatives of 4-dimethylaminoazobenzene tested in our previous work (7) give values of 4′—CH_3— = <1, 3′—CH_3— = 10—12, 2′—CH_3— = 2—3, and 2—CH_3— = 0—<1. The calculated value for the difficultly obtainable 3-CH_3— derivative was zero. An attempt was made to evaluate the relative importance of the position of a group in contrast to its type. For this purpose the effects of substituting HO—, Cl—, and NO_2— groups in the 4′, 3′, and 2′ positions were determined. Since all of the monohydroxy derivatives were inactive in the time interval studied the relative effect of this group in various positions remains unknown. However, a comparison of the carcinogenic activities of the corresponding methyl, chloro, and nitro derivatives yields some light on the problem of the position *versus* the type of substituent. In each series the 3′ derivative was the most active although the 3′-CH_3— derivative was about twice as active as the corresponding chloro and nitro compounds. Apparently, however, the 3′-nitro-4-dimethylaminoazobenzene which is absorbed is more active than 4-dimethylaminoazobenzene or its 3′-chloro derivative, since the observed carcinogenic activity of 5 is increased to 8–9 when a correction is included for the 40 per cent of the ingested dye which is excreted in the feces. Also in each case the 2′ derivative was the next most active compound; while each of these derivatives was of about equal activity, their relative activities were about a third of that assigned to the parent dye. Similarly the 4′ derivatives were the least active in each series although here the 4′-Cl compound was definitely more active than the 4′-CH_3 derivative. Here again, the difference in activity may be due partially to the excretion of 15 per cent of the administered 4′-methyl-4-dimethylaminoazobenzene in the feces. Unfortunately the 4′-NO_2 derivative was not tested because of its poor absorption from the tract. Hence from the limited data obtained so far it appears that the activities of monosubstituted derivatives of 4-dimethylaminoazobenzene on the prime numbered ring are likely to bear the relationship 3′ > 2′ > 4′ to one another; *i.e.*, the position of a group may be more important than its type. Three of the 6 possible dimethyl derivatives of 4-dimethylaminoazobenzene on the prime numbered ring were tested also. None were active. It is of interest that 2 methyl groups in the equiv-

alent 3′, 5′ positions produced an inactive compound although the presence of 1 methyl group in this position greatly enhances the activity of the parent dye. The 3′-ethoxy derivative was chosen as an example of a compound with a type of group in this high activity position which, assuming free rotation, would project out from the plane of the benzene ring a large share of the time; this specific alteration greatly lowered the activity of the molecule. Recently Sugiura (28) noted that the activities of the 2′-CH_3— and 3′-CH_3— derivatives of 4-monomethylaminoazobenzene corresponded closely with the activities of the same derivatives of 4-dimethylaminoazobenzene already reported (7). The activities of the 3′-CH_3— and 3-CH_3 derivatives of these two dyes (Table III) provide confirmation and extension of the general finding that *N*, *N*-dimethyl- and the corresponding *N*-monomethylaminoazo dyes are of equal activity.

The fact that the character of the diet may alter greatly the carcinogenic activity of an aminoazo dye is a well known and much explored problem (17). Much of the earlier work by the Japanese (8, 20) on the comparative activity of azo dyes was performed with a diet consisting largely of rice; our objections to the use of this diet have been voiced previously (7, 17). The rice diet has also been used in this country for structure studies (9, 28) although with better survival of the rats than the Japanese experienced. However, the report by Kensler *et al.* (19) that the rice diet causes a rapid demethylation of 4-dimethylaminoazobenzene *in vitro* provides still another strong reason for discarding the use of this diet in many azo dye experiments. No destruction (29) or demethylation (30) of azo dye has been found to occur in any of our diets. In this connection it should be noted that Sugiura (28) recently found that 4-ethylmethylaminoazobenzene possessed an activity of 1+ on his scale of activity while 4-dimethylaminoazobenzene was assigned a rating of 2+. However, under our conditions these two compounds possessed equal activity. It is possible that the discrepancy might be explained on the basis of a dealkylation of the ethylmethyl dye in the rice diet since this compound might be partly demethylated to the inactive dye, 4-monoethylaminoazobenzene, prior to feeding. Deethylation in the diet, which would produce the active 4-monomethylaminoazobenzene, is unlikely since a lower activity was found and also because 4-diethylaminoazobenzene is stable in the rice diet (19).

While an attempt has been made in this work to measure the carcinogenic activity of several aminoazo dyes relative to that of 4-dimethylaminoazobenzene, it must be emphasized that only *rough* figures for these relative activities under one set of conditions have been obtained. It is probable that the same comparisons made under other conditions would yield different values. For example, it is established that 3′-methyl-4-dimethylaminoazobenzene is less affected by dietary alterations than is 4-dimethylaminoazobenzene (11); hence, for example, a comparison of activity of these two dyes using a diet

high in riboflavin would almost certainly lead to a higher relative activity for the 3′-methyl derivative than was found with diets low in riboflavin. The exceptional case of the effect of a diet on a carcinogen *in vitro* and the uncertainty introduced by a poorly absorbed test compound have been considered above. Differences in the strain and species of the test animals are still other factors that might alter the relative activities of carcinogens. The reversal of the relative activities of 4-dimethylaminoazobenzene and 2′, 3-dimethyl-4-aminoazobenzene ("*o*-aminoazotoluene") in the rat and mouse (23) is a case in point. However, greater difficulties are met in considering whether a given relative activity is really true. For example, we have pointed out (7) that if carcinogens undergo metabolic reactions unrelated to the carcinogenic process, the number of tumors obtained in a given experiment is not necessarily a true index of the carcinogenicity of the molecule tested. Activity tests of compounds structurally related to carcinogens are important first steps and often yield new carcinogens and other useful starting points for further research. But any attempt to deduce the nature of a carcinogenic process solely from the activities of a series of structurally related compounds is of questionable validity. Accordingly, we are also investigating the mechanism of carcinogenesis by 4-dimethylaminoazobenzene through studies on the chemical and physical interactions of the dye with the constituents of the liver (1).

SUMMARY

1. Eighteen known or possible metabolites of the hepatic carcinogen 4- (or *p*-) dimethylaminoazobenzene were tested for carcinogenic activity in the rat. Of these compounds only 4-monomethylaminoazobenzene, a known metabolite, proved to be active. Eight compounds, which appear to be metabolites of the dye, were inactive; these included 4-aminoazobenzene, 4′-hydroxy-4-monomethylaminoazobenzene, 4′-hydroxy-4-aminoazobenzene, *N*-methyl-*p*-phenylenediamine, *p*-phenylenediamine, aniline, *p*-aminophenol, and *o*-aminophenol. Nine compounds which may possibly be metabolites also were inactive; these compounds were 4′-hydroxy-, 3′-hydroxy-, and 2′-hydroxy-4-dimethylaminoazobenzene, 4-formylaminoazobenzene, 4-hydroxyazobenzene, 2, 4′-diamino-5-dimethylaminodiphenyl, 3-dimethylaminocarbazole, *N*, *N*-dimethyl-*p*-phenylenediamine, and *p*-hydroquinone. A mixture of 9 known and possible metabolites was also found to be inactive. These data indicate that the primary carcinogen operative in tumor formation by 4-dimethylaminoazobenzene is probably an azo dye closely related to the parent carcinogen. This conclusion is supported by recent work from this laboratory which indicates that the primary carcinogen consists of either or both of the protein-bound dyes found in the liver, *i.e.* 4-monomethylaminoazobenzene and an unidentified polar aminoazo dye, and that the formation of bound dye constitutes one of the first steps in this carcinogenic process.

2. The carcinogenic activities of 19 other compounds related to 4-dimethylaminoazobenzene were tested to obtain more information on the structural features needed for a 4-aminoazo dye to possess *strong* activity in the rat. 3'-Methyl-4-monomethylaminoazobenzene and the corresponding dimethylamino derivative were nearly twice as active and 4-ethylmethylaminoazobenzene had the same activity as 4-dimethylaminoazobenzene. As tested 3'-nitro- and 3'-chloro-4-dimethylaminoazobenzene both had about the same activity as 4-dimethylaminoazobenzene; however, since the 3'-nitro derivative was incompletely absorbed its real activity appears to be about 1½ times that of 4-dimethylaminoazobenzene. 2'-Nitro- and 2'-chloro-4-dimethylaminoazobenzene were about one-half to one-third as active and 4'-chloro-4-dimethylaminoazobenzene was approximately one-fourth as active as the parent dye. 3'-Ethoxy-4-dimethylaminoazobenzene and 3-methyl-4-monomethylaminoazobenzene exhibited only slight carcinogenic activity. The following compounds proved inactive: the benzamide of *N*,*N*-dimethyl-*p*-phenylenediamine; the diethyl, monoethyl, benzylmethyl, β-hydroxyethylmethyl, and formyl derivatives of 4-aminoazobenzene on the amino group; and the 3-methyl, 3', 5'-dimethyl, 2',5'-dimethyl, and 2',4'-dimethyl derivatives of 4-dimethylaminoazobenzene. From the available data two conditions appear to be essential if a dye is to possess high activity: (1) at least one methyl group must be attached to the amino group together with the proper second substituent, and (2) the rings must bear either no substituents or carry only certain substituents, preferably in the 3' position.

3. The data on the carcinogenicity of the 2'-, 3'-, or 4'-methyl, chloro, and nitro derivatives of 4-dimethylaminoazobenzene show that the position of these groups determines the carcinogenicity of these compounds to a greater extent than does the type of group. The activity relationship was 3' > 2' > 4'.

4. Primary, secondary, and tertiary aminoazo dyes were determined in the livers and blood of rats fed aminoazo dyes which differed in the substituents on the amino group. The data show that deethylation of 4-diethyl-, 4-monoethyl-, and 4-ethylmethylaminoazobenzene occurs *in vivo* just as 4-dimethyl- and 4-monomethylaminoazobenzene are demethylated *in vivo*. However, 4-benzylmethylaminoazobenzene and 4-β-hydroxyethylmethylaminoazobenzene were dealkylated only slightly under similar conditions.

5. The following new compounds are described: 4-ethylmethyl-, 4-monoethyl-, 4-benzylmethyl-, and 4-β-hydroxyethylmethylaminoazobenzene; 4'-hydroxy-, 3-methyl-, and 3'-methyl-4-monomethylaminoazobenzene; 2'-hydroxy-, 3'-hydroxy, 3-methyl-, 3'-ethoxy-, 3',5'-dimethyl-, 2',5'-dimethyl-, and 2',4'-dimethyl-4-dimethylaminoazobenzene.

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THE INFLUENCE OF AVAILABLE FLUID ON THE PRODUCTION OF EXPERIMENTAL HEMOGLOBINURIC NEPHROSIS IN RABBITS

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It has been reported in a previous paper that hemoglobinuric nephrosis can be consistently produced when rabbits are deprived of water prior to repeated intravenous injections of hemoglobin (1). Since dehydration favors the precipitation of pigment casts in the kidney tubules of rabbits given hemoglobin, studies of fluid changes in which sodium thiocyanate dissolves before and after dehydration seemed desirable. Such studies might indicate whether there is a relationship between changes in available fluid and the occurrence of hemoglobinuric nephrosis.

Method

Animals.—Male and female rabbits from pedigreed New Zealand White stock weighing 2500 to 4200 gm. were used.

Preparation of Hemoglobin.—The original method of preparation of hemoglobin was modified by the addition of ether equivalent to 1 per cent of the total volume of water and centrifuged erythrocytes. This was done to delay alteration of the hemoglobin when solutions had to be kept as long as 1 week at 4–6°C. Following the last injection, the absorption properties of the hemoglobin were determined at different wave lengths in a Coleman spectrophotometer to determine whether alterations had occurred.

State of Hydration and Method of Injection.—Water and rabbit pellets[1] were withheld for 3 days. During the following 3 days each animal received 9 or 10 intravenous injections of hemoglobin which totaled 1.8 gm./kg. During the injection period the animals were not given any food. The morning following the last injection and for 6 days thereafter the rabbits were given 30 ml./kg. of drinking water and food as desired. After the 7th day water consumption was not restricted. From the 3rd to the 6th day the control group of rabbits received water to drink equivalent to the volume given intravenously to the test group.

Blood Studies.—The method of Crandall and Anderson (2) for the determination of available fluid was modified to make the $Fe(SCN)_3$ determinations in a Coleman spectrophotometer at 460 mμ. The fluid measured by sodium thiocyanate will hereafter be referred to as available fluid as recommended by Gregersen and Stewart (3).

The non-protein nitrogen (NPN) determinations were done before dehydration and on the 8th day after the initiation of the experiment.

Urine Studies.—In 7 test rabbits the daily output of urine, specific gravity, urinary pH, and the reaction of urine to heat and 5 per cent acetic acid were recorded.

Gross Observations and Histologic Studies.—The animals which died were autopsied and studied as previously described (1). The rabbits which survived were subjected to surgical

[1] Master mix rabbit pellets (McMillen Feed Mills, Division of Central Soya Company, Inc., Fort Wayne).

removal of the left kidney 11 to 16 days after the initial injection of hemoglobin. The kidney which was removed adequately demonstrated the degree of pigment cast accumulation since the lesions are uniformly bilateral. These animals, which had been operated upon, will be studied subsequently to determine the end results of hemoglobinuric nephrosis.

TABLE I

The Effect of Reduction in Available Fluid on the Development of Hemoglobinuric Nephrosis

Rabbit No.	Sex	Normal			After dehydration			Added observations in control and test groups			
		Weight	Available fluid volume	Per cent of body weight	Per cent of body weight	Weight loss	Total hemoglobin injected	Highest NPN recorded	Gross*	Combined kidney weight	Pigment casts observed‡
		gm.	*ml.*			*gm.*	*gm./kg.*	*mg. per cent*	*days*	*gm.*	
1	M	4225	1370	32.3	22.6	563	0	47	7	20.0	—
2	M	3442	1180	35.4	30.3	429	0	45	7	15.0	—
3	M	3361	1243	36.9	30.6	239	0	47	7	18.0	—
4	F	3616	1057	29.2	28.8	467	0	50	6	20.5	—
5	M	3958	1233	31.1	24.1	412	0	49	7	17.0	—
6	M	2584	875	32.9	21.2	254	1.8	300+	7§	30.5	3+
7	M	3441	1370	38.9	25.2	378	1.8	300	8§	38.6	3+
8	M	2934	882	30.1	22.0	262	1.8	51	17	17.0	2+
9	M	3457	1310	39.2	25.3	331	1.8	300+	7§	28.8	3+
10	M	3122	968	31.0	27.2	294	1.8	82	14	20.2	3+
11	M	4059	1290	31.7	26.4	353	1.8	72	19	22.4	2+
12	M	3225	1103	35.0	29.2	260	1.8	67	16	20.2	2+
13	F	3017	910	30.2	28.1	382	1.8	53	19		2+
14	F	3297	1005	31.8	26.8	431	1.8	49	14	16.4	1+
15	F	4025	1110	27.6	23.6	264	1.8	124	16	19.8	2+
16	F	3772	867	23.5	21.1	339	1.8	300+	8§	31.2	3+
17	F	3527	1212	34.4	31.4	327	1.8	56	16	17.6	2+
18	F	4097	982	24.0	23.9	357	1.8	265	6§	37.0	3+
19	F	3870	955	24.7	23.6	371	1.8	260	19	23.6	3+
20	F	3148	784	24.8	24.4	244	1.8	48	19	14.8	1+

* Includes 3 days of water deprivation.

‡ 1+ = less than one cast; 2+ = 1 to 5 casts; 3+ = 5 to 15 casts; average of 10 low power fields.

§ Rabbit died in uremia.

Results of Water Deprivation and Intravenous Injections of 1.8 Gm./Kilo of Hemoglobin

The significant data obtained on 5 control and 15 test rabbits are tabulated in Table I. Examination of column 4 reveals that there is a wide variation of available fluid in different animals. The 9 female rabbits exhibited still greater variability; 5 of these (Nos. 15, 16, and 18 to 20) had persistently low per cent fluid volumes in repeated tests. Such low available fluid volumes were not

observed in male rabbits. Gregersen and Stewart (3) in similar studies found mongrel female dogs usually had more available fluid and exhibited wider individual fluctuations than the males. They pointed out that since humidity, water intake, age, diet, and breed had not been controlled, the cause for the variation in available fluid remained unknown. In our study, all of the rabbits were of the same stock and approximately the same age; they were kept under direct observation in separate cages during the experiment, at which time they had adequate quantities of water and ate the same food. In spite of these controlled conditions, female rabbits normally exhibited wider individual fluctuations in their available fluid, and some of these had less fluid than the males.

Study of columns 5 and 6, in which the available fluid volume is expressed as percentage of body weight before and following dehydration, reveals that the rabbits respond differently to water deprivation. Eleven rabbits (Nos. 2 to 5, 10 to 15, and 17) with optimal initial values suffered minimal to moderate depletion of available fluid. Eight of these received hemoglobin and did not develop a fatal hemoglobinuric nephrosis. Five rabbits (Nos. 1 and 6 to 9) with optimal quantities of fluid initially sustained an excessive depletion (in excess of 8 per cent). Three of these 4 which received hemoglobin died from hemoglobinuric nephrosis. Four female rabbits (Nos. 16 and 18 to 20) with low available fluid volumes initially had a minimal to no depletion during dehydration. In this group 2 died of hemoglobinuric nephrosis, one developed an NPN of 260 mg. per cent and recovered, whereas the last rabbit was apparently unaffected. Two (Nos. 8 and 20) of 8 rabbits, therefore, with low available fluid volumes following dehydration did not develop fatal hemoglobinuric nephrosis or a significant elevation of NPN.

There is a good relationship between the combined kidney weight and the animals which died in uremia. The combined weight of the kidneys of rabbits dying of hemoglobinuric nephrosis is in excess of 28 gm. The kidneys from neither the control nor the test rabbits with transient hemoglobinuric nephrosis ever equaled this weight (column 11). Column 12 indicates that pigment casts were demonstrable in all of the test animals 3 to 16 days after the injection of hemoglobin. In this group 5 of 15 rabbits died of fatal hemoglobinuric nephrosis (column 10). Of the 10 rabbits which survived, all were able to withstand a nephrectomy 11 to 16 days following injections of hemoglobin without any apparent deleterious effect.

The postmortem findings generally agreed with those previously reported (1). An additional finding of considerable interest was minimal to extensive central necrosis of the liver, observed in 3 of 5 rabbits dying in uremia. None of the control rabbits had necrosis of the liver. Only 1 rabbit (No. 9) had increased amounts of pigment in the liver. There was vacuolar degeneration of liver cells in 5 rabbits which died in uremia and in 2 control rabbits. Pulmonary edema was seen in only 3 rabbits, and these died in uremia. Focal necrosis of tubular epithelium was present in 5 rabbits which died in uremia, in 1 which survived, and in none of the control group. Swollen epithelial cells with a coarsely granular cytoplasm were

present in 5 rabbits which died and in 3 which survived. Dilatation of proximal convoluted tubules was present in 3 and absent in 2 rabbits which died. Dilatation was also evident in 6 of the animals which survived, and to a minimal degree in 1 of the control rabbits.

Urinary Changes Observed.—Urine studies were made on 7 of 15 test rabbits and are tabulated in Table II. When the rabbits were allowed water and rabbit pellets at desire, the reaction of the urine was always alkaline. During the dehydration period the urine output decreased. The specific gravity and pH changes were variable. Only 2 of 7 rabbits developed an acid urine. No protein was found under normal conditions or during dehydration. Following the injections of hemoglobin, 2 rabbits developed anuria, 5 rabbits continued to

TABLE II

Urine Changes Observed Following Food and Water Deprivation and Intravenous Injections of Hemoglobin

Rabbit No.	Sex	Control for 3 days			Water deprivation for 3 days			Hemoglobin injected for 3 days			
		Total urine output	Lowest specific gravity	Lowest pH	Total urine output	Lowest specific gravity	Lowest pH	Total urine output	Lowest specific gravity	Lowest pH	Proteinuria
		ml.			*ml.*			*ml.*			
6	M	250	1.040	8.2	181	1.025	6.9	0	0	0	0
7	M	456	1.030	8.2	135	1.057	8.1	0	0	0	0
8	M	261	1.030	8.2	130	1.047	6.6	111	1.037	5.6	+
10	M	371	1.035	7.8	94	1.030	7.8	180	1.026	6.0	+
11	M				234	1.034	7.9	198	1.024	5.7	+
15	F	305	1.038	8.2	95	1.042	8.2	249	1.012	5.9	+
16	F				291	1.037	7.7	306	1.013	5.7	+

Rabbit numbers correspond to those given in Table I.

urinate. One of these 5 rabbits died in uremia (No. 16). Comparison of the urinary output columns during dehydration and after injections of hemoglobin shows that in 3 animals more urine was excreted following hemoglobin injection. The specific gravity after hemoglobin is usually lower than in the previous periods. In 2 rabbits (Nos. 15 and 16) the specific gravity dropped to 1.013 and 1.012. One of these rabbits survived whereas the other died in uremia. During the period of hemoglobin injection all of the rabbits which urinated had an acid urine and a proteinuria

DISCUSSION

There is close agreement of the postmortem findings in this experiment with those previously observed (1). Additional findings, not previously described, are central necrosis and vacuolar degeneration of the liver as well as pulmonary edema. The conditions of the first experiment are not comparable to this

study, since both the dosage of hemoglobin given and the period of water deprivation varied in different rabbits. The arbitrary conditions in this experiment are more severe and were identical for each rabbit. The shorter survival periods in those animals which died, the finding of central necrosis of the liver, and pulmonary edema are probably due to the modifications which were introduced.

Tubular dilatation was observed in 3 rabbits in the previous report and in 3 rabbits in this experiment which died in uremia. The presence of tubular dilatation probably indicates a mechanical obstruction and also, that filtration of fluid into the renal tubules persists for a period thereafter. Uremia which develops in such animals might be wholly attributed to the blockage of the tubules. However, the observation in 2 of 5 rabbits, in the present study, in which death occurred without tubular dilatation, suggests that other mechanisms besides mechanical blockage of renal tubules are probably functioning in hemoglobinuric nephrosis. Apparently, therefore, the widespread degeneration of tubular epithelium and the foci of tubular necrosis play a greater rôle in the production of uremia than was originally suspected.

Bywaters and Stead (4) and Flink (5) have stressed the importance of the quantity of hemoglobin injected in the production of hemoglobinuric nephrosis. In the present study a constant quantity of hemoglobin (1.8 gm./kg.) was purposely used. The results which were obtained in 15 test rabbits adequately demonstrate that in addition to the quantity of hemoglobin given, which unquestionably exerts a direct influence on the production of this syndrome, other factors of equal importance are functioning simultaneously. One factor of importance during hemoglobinemia is the presence of adequate reserves of available fluid in the organism. Flink (5) has demonstrated that intravenous injections of hemoglobin into dogs not deprived of water always produced a hemodilution. Whether the hemodilution resulted from the hemoglobin or other compounds formed during the hemolysis of erythrocytes is not known. Nevertheless, this is probably one of the means by which the organism hastens the elimination of hemoglobin. In the urine studies intravenous hemoglobin solutions were observed to exert a diuretic effect in 3 of 7 rabbits. However, when the available fluid compartment is first depleted sufficiently by water deprivation, the elimination of hemoglobin is either delayed or completely inhibited. Under such circumstances, then, the hemoglobin enters the kidney tubules, pigment casts are formed, degeneration and focal necrosis of tubular epithelium appear, and uremia follows.

Keith (6) and Elkinton and Taffel (7) demonstrated that water deprivation exerts pronounced physiologic alterations. Yorke and Nauss (8) originally pointed out that feeding dry oats to rabbits first increased the severity of hemoglobinuric nephrosis after intravenous injections of hemoglobin. Bywaters and Stead (4) by combining dehydration with acid diets and compres-

sion of leg muscles in rabbits, prior to injections of human myohemoglobin, were able to produce myohemoglobinuric nephrosis in their animals. Rigdon and Cardwell (9) found water deprivation in rabbits enhanced their susceptibility to sucrose nephrosis. In the present study it was possible to demonstrate a close relationship between the depletion of available fluid and the occurrence of hemoglobinuric nephrosis in 13 of 15 rabbits. Two rabbits with available fluid volumes in the critical range after water deprivation survived. The findings in these animals suggest that other factors besides available fluid volume are important in the elimination of hemoglobin by the organism. The mechanism by which the depletion of the available fluid exerts its influence on the production of this syndrome remains to be determined.

SUMMARY

The importance of previous dehydration on the production of hemoglobinuric nephrosis is substantiated. Hemoglobinuric nephrosis regularly occurred in rabbits 3 to 16 days following the injections of hemoglobin. Five of 15 animals died of fatal hemoglobinuric nephrosis; the combined kidney weight in these exceeded the weight of the kidneys of control rabbits and of those which survived. Additional observations, not previously made, are focal necrosis of the liver and pulmonary edema in some of the rabbits which died. A relationship was evident between the quantity of available fluid and the severity of the hemoglobinuric nephrosis which developed after injections of hemoglobin.

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RENAL ATHROCYTOSIS AND INTRACELLULAR DIGESTION OF INTRAPERITONEALLY INJECTED HEMOGLOBIN IN RATS

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PLATES 3 TO 5

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During the past five years studies on proteinuria induced in rats by the intraperitoneal injection of various proteins have been carried on in the laboratory of the Renal Clinic at Stanford Hospital. The integrating ideas behind the design of the studies were that the undamaged glomerulus of the rat is normally permeable to small amounts of protein; that in the course of the formation of the large daily glomerular filtration volume a relatively large amount of protein is filtered through; that some reabsorption of the protein takes place along the course of the renal tubule; that the renal epithelium can reabsorb a certain maximum amount; and that, therefore, proteinuria will result if the renal epithelium is overloaded with protein. These studies were carried on without systematic histological investigation of the available material. It is the purpose of the present paper to report some of the preliminary findings, the primary concern here being to trace the fate of the injected hemoglobin with the aid of histological and histochemical techniques.

The notion of glomerular filtration and tubular reabsorption of large molecules, including proteins, is not new. Athrocytosis has been described by Gérard (1) as a "characteristic property of the vertebrate nephron" arising after the absorption of electro-negative colloids through the apical surfaces of renal epithelium, followed by intracellular "flocculation" of the absorbed material. The term athrocytosis, according to Lison (2), was first used by Burian, later by von Mollendorf, then resurrected, after a period of disuse, by Gérard and Cordier (1); it is equivalent to the German term *Speicherung*. Randerath (3), in a comprehensive survey of the histogenesis of the various forms of nephritis, has reviewed the work of recent German and Belgian authors. He concludes that in "nephrosis" altered permeability of the glomerular capillaries permits the passage of large amounts of protein which produces the characteristic histological alterations; *i. e.*, thickening of the glomerular basement membrane and hyaline droplet formation in the renal epithelium, the latter being the visible evidence of athrocytosis or *Speicherung*.

The human glomerular membrane is usually considered to be impermeable to protein, for all practical purposes, by most authors in this country. Passage of protein, indicated by its appearance in the urine, is taken as evidence of damage to the glomerular "filter." However, Oliver (4) has recently reviewed certain considerations which

strongly favor the view that protein molecules are continually passing into the glomerular space and being reabsorbed (athrocytosed) by the epithelium of the proximal convoluted tubules. These considerations are: (1) mammalian glomerular fluid cannot be called protein-free by actual test, it can only be said to contain less than 25 mg. per 100 cc. of filtrate; (2) if the concentration of protein in the glomerular filtrate were only one one-thousandth of its concentration in the plasma, *i. e.* about 7 mg. per 100 cc., a proteinuria of 0.6 gm. per 100 cc. would result, in the absence of tubular reabsorption; (3) exertion, even moderate, is commonly followed by the appearance of protein in the urine; furthermore Oliver has presented histological evidence of the reabsorption from the tubular urine of gelatin, gelatin polymer, horse serum albumin, and calcium gelatinate by the cells of the proximal convoluted tubules in rats. There was no evidence that the glomerular membranes were damaged prior to, or by, the passage of these large molecules.

If such filtration and reabsorption does occur in the mammalian kidney, under physiological circumstances, as seems likely, there must be some mechanism in the renal epithelium for disposing of the protein. If even only a very small fraction of the plasma protein were filtered out in any one circuit of the blood, the formation of approximately 180 liters of glomerular filtrate a day, as in the human kidney, would yield a considerable quantity of protein which could not be stored indefinitely. In this paper hemoglobin is studied as an example of how such protein might be handled.

Smetana (5, 6) has studied the kidneys of urodeles, mice, rats, guinea pigs, rabbits, and dogs, using various proteins linked with the disodium salt of 2-naphthol-3:6-disulfonic acid. This firmly linked protein-dye complex was prepared from crystalline egg albumin, dog serum albumin, casein, and peptone. It was given intravenously to the test animals. He was able to demonstrate brightly colored droplets of the complex in the renal epithelium of the proximal convoluted tubules in urodeles, rats, mice, and dogs, but was not able to find them in guinea pigs and rabbits. In the urodeles the droplets showed no signs of splitting or digestion and were present as late as 328 days after injection. Smetana concluded that such reduction in number as did occur was due to desquamation of entire cells, or due to the discharge of particles from the tubular lining cells through tiny pores in the basement membrane, after which they were taken up by phagocytic stromal cells. In the other animals he found widespread storage of the protein-dye complex in the reticulo-endothelial cells of the viscera as well as in the renal epithelium, but again there was no evidence of digestion or disposal of the material.

Smetana's protein-dye complex behaves much like a foreign body and gives us little insight into the nature of a process by which the kidney may be able to deal with athrocytosed protein. Possibly his compound was denatured and in some way resistant to enzyme action. The protein-dye complex was toxic and it was possible to give only relatively small doses. Hemoglobin, on the other hand, can be injected intraperitoneally in large doses into rats without ill effect. Moreover, hemoglobin has a natural "marker" prosthetic group which gives it a distinctive color and staining reaction, making its identification in the tissues easy. As a further advantage its breakdown can be studied by the histochemical demonstration of released iron.

Materials and Methods

Rats, of approximately 150 gm. body weight, from the colony maintained at Stanford Medical School by Dr. Addis, were taken off stock diets and limited to 10 per cent dextrose *ad libitum* during the course of the experiments. Purified human hemoglobin,[1] prepared by the method of Hamilton and Farr (7), was injected intraperitoneally twice daily, at 4 p. m. and 7 a. m. A complete course of injections consisted of five doses of 16 cc. each. The hemoglobin solution injected[2] contained 6.2 gm. per 100 cc. and was made up freshly from a stock solution, standardized colorimetrically at frequent intervals, containing 9.6 gm. per 100 cc. Thus the rats, on the average, received 5 gm. of hemoglobin (33 gm. per kilo) if given the complete course. A single injection of 1 gm. amounted to 6.7 gm. per kilo. As shown in the tables the urine output per hour, excretion of hemoglobin in the urine and serum hemoglobin were determined,[3] and the rats were killed, by exsanguination from the aorta under ether anesthesia, at varying intervals after the first injection. In the first experiment (Table I) the kidneys were weighed and fixed in 10 per cent formalin together with liver slices. From paraffin blocks sections were cut at 8 micra and stained with hematoxylin and eosin, azocarmine, and Dunn's (8) hemoglobin stain. Iron released by the intracellular breakdown of hemoglobin was identified histochemically by a Berlin blue technique. The modification of the method used here involved the immersion of the section, for as long as 25 minutes, in a freshly prepared solution made by mixing one part of 2 per cent potassium ferrocyanide with three parts of 1 per cent hydrochloric acid. The pigment thus identified is referred to as hemosiderin.[4] In addition to the preparations mentioned, unstained formalin-fixed sections 1 or 2 mm. thick were cleared by the Spalteholz technique (10) and were examined under the dissection microscope. In this way it is possible to observe the disposition of intraluminal "casts" in a manner impossible with routine sections. In the second experiment (Table II) spleen and femoral marrow in addition were fixed in 10 per cent formalin and studied. *Note on the cytology of rat proximal convoluted tubular epithelium:* In the colony of rats used in these experiments the renal epithelium of the proximal tubules, as studied in formalin-fixed preparations stained with hematoxylin and eosin, is devoid of particulate elements except for a very few hyaline droplets, seen only after careful study with the oil immersion lens. Very rarely an intracellular droplet of yellowish brown pigment, which may or may not give the Berlin blue reaction is found. All of the rats show a moderate degree of phagocytosis of hemosiderin in the spleen. These changes are attributable to latent *Bartonella* infections, to which the rats will succumb if splenectomized. It has been observed in control tissues of rats from other colonies examined in this laboratory that, in association with advanced hemosiderosis of the spleen, large numbers of pigmented bodies may be found in the proximal convoluted tubular epithelium. Some of these give the Berlin blue reaction, others do not. They do not have

[1] Furnished by Dr. R. B. Pennell of Sharp and Dohme, Inc. This work was aided in part by Grant R.G.85 from the U. S. Public Health Service.

[2] Osmometric determinations of molecular weight on samples of the solutions injected, carried out at the California Institute of Technology under the direction of Dr. Linus Pauling, gave results between 62,000 and 68,000.

[3] These determinations were carried out by Miss Jan Ureen of the Renal Clinic.

[4] Lison (9) points out that the *in vitro* decomposition of hemoglobin into a globin and hematin, thence into hematoporphyrin and iron does not occur *in vivo*. Here the normal process of breakdown gives rise to an iron-containing pigment, hemosiderin, and another iron-free pigment. Hemosiderin has been considered as an albuminate of iron, formed by the union of ferric or ferrous iron with a protein. Recently, however, isolated granules of the pigment have been analyzed and found to consist of ferric hydroxide largely, with about 25 to 36 per cent of protein and traces of calcium phosphate.

the staining reactions of hemoglobin. Such rats, however, would be unsuitable for studies of the type described in this paper.

Experiment 1 (Table I)

Histological and Histochemical Findings.—2 hours after the first injection: in the hematoxylin and eosin preparation there were visible a few rounded, discrete, reddish bodies, aver-

TABLE I

No. of rats	Time after first injection	Average urine volume	Average urinary excretion of hemoglobin	No. of rats killed	Average serum hemoglobin	Combined kidney weight corrected to 150 gm. body weight
	hrs.	*cc./hr.*	*mg./rat/24 hrs.*		*mg./ 100 cc.*	*mg./rat*
Rats receiving one injection of 16 cc. of 6.2 per cent hemoglobin						
9	2	1.02	38.8	2	735	1010
7	4	2.15	453.3	2	950	958
5	8	0.84	462.0	2	1010	1110
3	17	1.53	515.0	3	575	1140
Rats receiving five injections of 16 cc. of 6.2 per cent hemoglobin						
9	15	1.60	533.0			
	18	1.68	658.0			
	21	0.75	665.0			
	24	1.00	755.0			
	39	1.09	859.0			
	42	1.70	653.0			
	45	1.49	1080.0			
	48	1.05	980.0			
	65	0.87	785.0	3	875	2160
6	69	0.39	128.0			
	72	0.25	66.8			
	89	0.17	13.3	1 died		
5	113	1.19	0	2	0	2413
3	139	2.22	0			
	161	2.92	0	1	0	1755
2	185	2.62	0	1	0	1842
1	209	1.75	0			
	233	1.87	0			
	257	2.09	0			
	329	2.38	0	1	0	1205

aging 1 to 2 micra in diameter, within the cells lining the proximal convoluted tubules. These bodies stained green with the hemoglobin stain, as did the erythrocytes. No iron was demonstrable by the Berlin blue technique, nor was there visible pigment. There was no hemoglobin in the glomerular spaces or within the lumina of the nephrons. *4 hours:* (Fig. 1.) The bodies had become more numerous and often lay in clear spaces about the nuclei. There was no intracellular iron demonstrable. *8 hours:* The bodies were still more numerous and in a few of the cells there were very small amounts of iron. Some intraluminal hemoglobin was

present. To judge from the appearance of the thick sections cleared by the Spalteholz technique this was in the connecting tubules and the terminal portions of the distal convoluted tubules. *17 hours:* (Figs. 2 and 3.) When examined at a magnification of 22 diameters the renal cortex in the hematoxylin and eosin–stained sections appeared diffusely orange, and with the hemoglobin stain diffusely greenish. This was seen under higher power to be due to enormous numbers of the intracellular bodies previously described. A few of these were as large as 4 micra in diameter. The amount of demonstrable intracellular iron had increased slightly, though it was difficult to discern pigment in the hematoxylin and eosin preparations. The amount of intraluminal hemoglobin had increased greatly. Examination of the thick cleared sections (Fig. 5) revealed that some of the nephrons were beautifully delineated by their content. The distal segment, loop of Henle, and the descending limb of the proximal segment were clearly visualized. The collecting tubules of the cortex and renal papilla were empty. No more than a few hundred of the total sixty thousand or so nephrons were delineated in this manner. In the liver the parenchymal cells were of normal appearance. There was no hemoglobin or demonstrable iron in either the parenchymal cells or the Kupffer cells. *65 hours:* The most striking change was the patchy distribution of the intracellular hemoglobin and the appearance of large quantities of intracellular hemosiderin (Fig. 4) in the proximal convoluted tubular epithelium. With the Berlin blue technique the outer cortex appeared a bright blue, as seen in slides examined without magnification. More hemosiderin than hemoglobin was present within the cells. In many of the glomerular spaces hemoglobin was present as a hyaline structureless coagulum with characteristic staining reactions. In the thick cleared sections it was apparent that a large number, possibly a majority, of the nephrons were filled in the manner previously described. The renal papilla was cleared and, as before, the collecting ducts were found to be free of intraluminal hemoglobin. A few loops of Henle (Figs. 6 and 8) dipped down almost to the tip of the papilla but were easily identifiable. In the *liver* there was no hemoglobin or demonstrable iron in the parenchymal or Kupffer cells. *113 hours:* The amount of intracellular hemoglobin had decreased greatly but there was a large amount of intracellular hemosiderin present. In various places in the cortex and medulla the tubular walls had broken down, hemoglobin within the lumina had been extruded, and about this extruded material were clustered polymorphonuclear leucocytes and a few giant cells. In the thick cleared sections it was apparent that most of the formerly filled nephrons had emptied, either completely or partially, leaving dilated, plugged, and tortuous segments (Fig. 7) behind. There was no hemoglobin or hemosiderin in the parenchymal or Kupffer cells of the liver. *161 hours:* Almost all demonstrable intracellular hemoglobin had disappeared from the renal epithelium, though there was still a large amount of hemosiderin. This pigment was seen in two situations: as rather fine droplets, less than 1 micra in diameter, within the cells of proximal convoluted tubules lined by columnar eosinophilic epithelium which appeared quite normal; and in coarser, more abundant intracellular masses within flattened basophilic epithelium which lined atrophic small tubules. Such atrophic tubules were uncommon. In the liver there was no significant alteration. *185 hours:* The number of filled segments seen in the cleared preparations had further decreased. There were many areas of cellular reaction to extruded hemoglobin in both cortex and medulla. Some of the atrophic tubules previously described were present. In the liver a few of the Kupffer cells contained small amounts of demonstrable iron. *329 hours:* Almost all of the proximal convoluted tubules were lined by normal appearing eosinophilic epithelium containing fine droplets of hemosiderin, clearly visible only with the oil immersion lens. There were a few clusters of atrophic tubules whose lining cells contained dense masses of hemosiderin. One flat depressed pigmented area seen on gross examination of the cortex proved to be a cluster of these tubules. In the thick cleared sections there were visible a few dilated, tortuous segments. There was no demonstrable intracellular hemoglobin. In the liver the amount of demonstrable iron in the Kupffer cells was perhaps slightly increased.

Experiment 2 (Table II)

As the table shows this experiment was designed so that larger numbers of rats would be killed at certain key times. Through an error some of these

TABLE II

No. of rats	Time after first injection	Average urine volume	No. of rats killed	Combined kidney weight
	hrs.	*cc./hr.*		*mg./rat*
Rats receiving one injection of 16 cc. of 6.2 per cent hemoglobin				
6	17	1.03	6	1052
Rats receiving two injections of 16 cc. of 9.6 per cent hemoglobin				
3	15	2.00		
	24	0.95	3	1082
Rats receiving two injections of 6.2 per cent and one 9.6 per cent				
3	15	1.63		
	24	0.72		
	42	1.30	3	1560
Rats receiving five injections of 16 cc. of 6.2 per cent hemoglobin				
12	15	1.88		
	24	0.96		
	42	1.08		
	51	1.15		
	65	0.92	6	1826
6	68	0.32		
	74	0.26		
	95	0.46		
	116	1.96		
	164	3.11		
	237	1.90		
	333	1.92	3	1311
3	405	1.90		
	501	2.13	3	969

animals received larger doses than had been planned. In this experiment spleen, liver, kidney, and femoral marrow were studied. The marrow was obtained by cutting both ends from the femur, inserting a large bore needle, attached to a syringe, into the medullary cavity, and blowing out the content. Little information was gained from the study of the spleens since a mild degree

of hemosiderosis was common to most of the rats in this colony. The only conclusion that could be drawn was that hemoglobin did not appear in the splenic phagocytes. In the following notes only such changes as conflict with, or add to, those described under Experiment 1 will be commented upon.

17 hours after the initial injection: No hemoglobin was demonstrable in the spleen, femoral marrow, or liver. There was no demonstrable iron in the femoral marrow. *24 hours:* There were huge numbers of intracellular hemoglobin bodies in the cells of the proximal convoluted tubules, and a small amount of demonstrable iron. Within a few cells the hemoglobin was in the form of a single, comparatively huge, body as large as 5 micra across. There was no hemoglobin in the liver, spleen, and femoral marrow. *42 hours:* These rats had received exceptionally large doses of hemoglobin. In both the thick cleared sections and in the routine sections it appeared as if a large majority of the nephrons had been filled in the manner previously described. A great many of the glomerular spaces contained dense, structureless sheets of hemoglobin. There was a large amount of demonstrable iron in the renal epithelium, traces in the Kupffer cells, but none in the femoral marrow. There was no hemoglobin in the liver, spleen, or femoral marrow. *65 hours:* Mitotic figures were prominent in the tubule cells of the boundary zone and some of the walls were disrupted. *333 hours:* More damage to the kidney was seen in these three rats than in the one comparable rat of Experiment 1. Scattered throughout the cortex were clusters of tubules, apparently from single nephrons, lined by low cuboidal epithelium and containing dense intracellular masses of hemosiderin. There were areas of interstitial inflammatory reaction in both cortex and boundary zone, some characterized by giant cells which had multiple nuclei and contained masses of hemoglobin. There were a few dilated tubular segments filled with unchanged hemoglobin and clustered about by polymorphonuclear leucocytes. The collecting tubules of the papilla appeared normal. Even in the cortex by far the great majority of the proximal convoluted tubules were of the usual size and staining reaction though there were fine particles of hemosiderin within most of the cells. *501 hours:* There were a few atrophic tubules but most appeared normal and fine particles of intracellular hemosiderin were fewer.

Experiment 3

Six rats were given a complete course of five injections of 16 cc. of 6.2 per cent hemoglobin and returned to stock diets. No urine or blood level studies were carried out on this group. Three were killed after 5 weeks. In the gross there were several depressed, brown, areas on both kidneys of two of the rats those of the third rat appearing normal in the gross. Microscopically the scars (Figs. 9 and 10) were seen to be formed by atrophic tubules surrounded by a lymphocytic infiltrate. Elsewhere there were fine particles of intracellular hemosiderin in the proximal tubular epithelium which otherwise appeared normal (Fig. 11). Intraluminal contents of any sort were practically absent and detectable only in the thick cleared sections. The remaining three rats were killed after 9 weeks. Of these only one had grossly visible scarring. Microscopically there were rare atrophic pigmented tubules. The remainder of the tubules were as before. A few intertubular phagocytic cells contained hemosiderin.

RÉSUMÉ AND DISCUSSION

Within 2 hours (Table I) the serum hemoglobin rose to a level of 735 mg. per 100 cc. The average urinary excretion of hemoglobin of nine rats was 3.23 mg. For comparison with other data in the table this has been calculated on a 24 hour basis, amounting to 38.8 mg. The comparatively low level of hemoglobin excretion during the first 2 hours is attributable to the time required for the absorption of hemoglobin from the peritoneal cavity into the blood stream. It is theoretically possible that a lag in the excretion of hemoglobin dependent on complete athrocytosis of the hemoglobin from the tubular urine might be demonstrated with appropriate doses, but such conclusions cannot be drawn from the figures here available. In the two rats killed at the end of the 2 hour period, discrete bodies, with the staining characteristics of hemoglobin, were found in small numbers within the epithelium of the proximal convoluted tubules. The weight of hemoglobin athrocytosed is too small for estimation from the combined kidney weights. The number of intracellular hemoglobin particles increased rapidly, until, in the three rats killed 17 hours after the initial injection, the renal epithelium was packed with them (Figs. 2 and 3). Here again it is useless to attempt a calculation of the weight of athrocytosed material since at this stage the intraluminal deposition of hemoglobin had become relatively prominent (Fig. 5). It can be said, however, that a significant formation of "casts" did not take place until the amount of athrocytosed material reached a maximum. The appearance of hemosiderin and the histochemical identification of iron with the Berlin blue technique was taken as evidence that intracellular splitting of hemoglobin had occurred. It would not be accurate to infer that there was a delay of 8 hours in this process. The breakup of the molecule probably occurred some time before the release of inorganic iron. This histochemically demonstrable iron reached its maximum in about 65 hours; thereafter it slowly disappeared in the manner described above. Hemoglobin was not at any time demonstrable in the parenchymal cells of the liver, the Kupffer cells, nor in phagocytic cells of the spleen or femoral marrow. Only a very tiny, almost negligible, amount of histochemically identifiable iron appeared in those sites.

Intraluminal Accumulation of Hemoglobin.—No attempt was made to alter or assess the pH of the urine during these experiments. Previous experience with the colony has shown that the rats excrete a urine with a pH of about 6 with great persistence, despite most dietary alterations. As mentioned above, the intraluminal deposition of hemoglobin was not prominent until the renal epithelium was packed with intracellular hemoglobin. At this stage, probably, the cells were unable to take in any more hemoglobin from the tubular urine. However this may be, the hemoglobin was observed to accumulate first in the terminal portion of the distal tubules or in the connecting tubules. The collect-

ing tubules remained free of hemoglobin. What occurred after this might be interpreted as a retrograde filling of the nephrons (Figs. 5 and 8). It appears that some of the proximal convolutions must have filled in this way. The interpretation of the dense sheet-like masses of hemoglobin in the glomerular spaces is otherwise obscure. Such masses of hemoglobin in the glomerular spaces indicate a concentration there greater than in the circulating blood. An alteration in permeability of the glomerular membrane alone could hardly account for this, nor do the data in Table I suggest that such permeability existed. With serial sections it was possible to show that the dense masses of hemoglobin in the glomerular spaces were continuous with a similar content of the proximal convoluted tubules (Fig. 12).

Structural Damage.—There was no evidence that the intracellular accumulation of hemoglobin damaged the renal epithelium. It was not until large amounts of hemoglobin were present within the lumina of the nephrons that changes indicative of injury became noticeable. These were the appearance of mitotic figures in the renal epithelium of the boundary zone, flattening of the epithelium of the tubules which contained intraluminal hemoglobin, rupture or dissolution of the tubular walls, extrusion of hemoglobin into the interstitial tissue, and inflammatory reaction, with or without giant cell formation. There were relatively few damaged nephrons at the termination of the experiments. These were of two types: one with a dilated lumen plugged with apparently unchanged hemoglobin, sometimes clustered about by leucocytes; the other an atrophic nephron whose proximal convolution was lined by small low cuboidal, rather basophilic, cells which contained dense masses of hemosiderin. Azocarmine stains showed no alteration in the thickness of the glomerular membranes, either during or after the passage of hemoglobin.

Oliguria.—In both experiments oliguria occurred near the 70th hour after the initial injection and about 20 hours after the final injection. The average urine output per hour dropped to about 15 per cent of its previous value. The tables indicate that a diuresis of short duration succeeded the oliguria at about the 110th hour after the initial injection. From the appearance of the thick cleared sections studied at the 65 hour stage (Fig. 6) it seems that the oliguria is to be explained as a consequence of interference with flow through the nephron, and was not a consequence of plugging of the collecting ducts. The great increase in the weight of the kidneys at this stage indicates the amount of intraluminal hemoglobin present.

It is clear that the experimental data contained in this paper neither confirm nor deny the hypothesis outlined in the beginning, though they are compatible with it. Evidence of renal athrocytosis of the intraperitoneally injected protein was found as early as 2 hours after the first injection. The total excretion of hemoglobin during the first 2 hours was relatively little, although at the close

of this period the blood level had risen to 735 mg. per 100 cc. The hypothesis, as outlined, implies an intracellular breakdown of the athrocytosed protein and its return to the blood stream in a simpler form. These experiments show that iron, capable of reacting to form ferric ferrocyanide, is detectable within 8 hours after the initial injection. The fate of the globin portion of the hemoglobin molecule is not traceable with the techniques used here.

With respect to the distribution of hemoglobin and, subsequently, of hemosiderin, it is of considerable interest that neither substance was found at any time after the initial injections in the Kupffer cells of the liver, in the femoral marrow, or in the spleen. It might have been expected that phagocytosis of hemoglobin and the formation of hemosiderin would have occurred at these three sites. The failure to find it is all the more puzzling when one considers that the concentration of hemoglobin in the plasma must have been greater than in the fluid in the proximal convoluted tubules.

The subsidiary findings in these experiments relate to the questions of oliguria as a consequence of hemoglobinemia, and the occurrence of structural damage to the kidney after the passage of large amounts of hemoglobin. With respect to the first question, we have shown that a transient oliguria may be a consequence of hemoglobinemia in the rat when the plasma level is in the neighborhood of 1 gm. per 100 cc., a level which would be approached in a man after hemolysis of about 400 cc. of blood. The oliguria in the rat seems to be due, not to a "plugging" of the collecting tubules, but to a simple interference with flow through the lumen of the nephron, presumably a consequence of the increased viscosity of the protein-rich fluid. With respect to the second question, we have shown that structural damage to the kidney does occur, but it is of moderate extent and severity, and the ultimate effect on the kidney is slight.

SUMMARY

The athrocytosis of hemoglobin by the epithelium of the proximal convoluted tubules in the kidney of the rat was studied after its intraperitoneal injection, absorption into the blood stream, and glomerular filtration. The earliest appearance of the athrocytosed particles, their accumulation, intracellular breakdown, and disappearance were followed. In addition observations were made on the intraluminal disposition of hemoglobin "casts" with the Spalteholz clearing technique; on the structural changes in the kidney in consequence of the injections; and on the development of a transient oliguria during the course of the experiment. The significance of the findings is considered.

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EXPLANATION OF PLATES

PLATE 3

FIG. 1. Experiment 1, 4 hours after intraperitoneal injection of hemoglobin. Formalin fixation, hemoglobin stain. There are a few intracellular particles of hemoglobin in the proximal convoluted tubular epithelium. × 357.

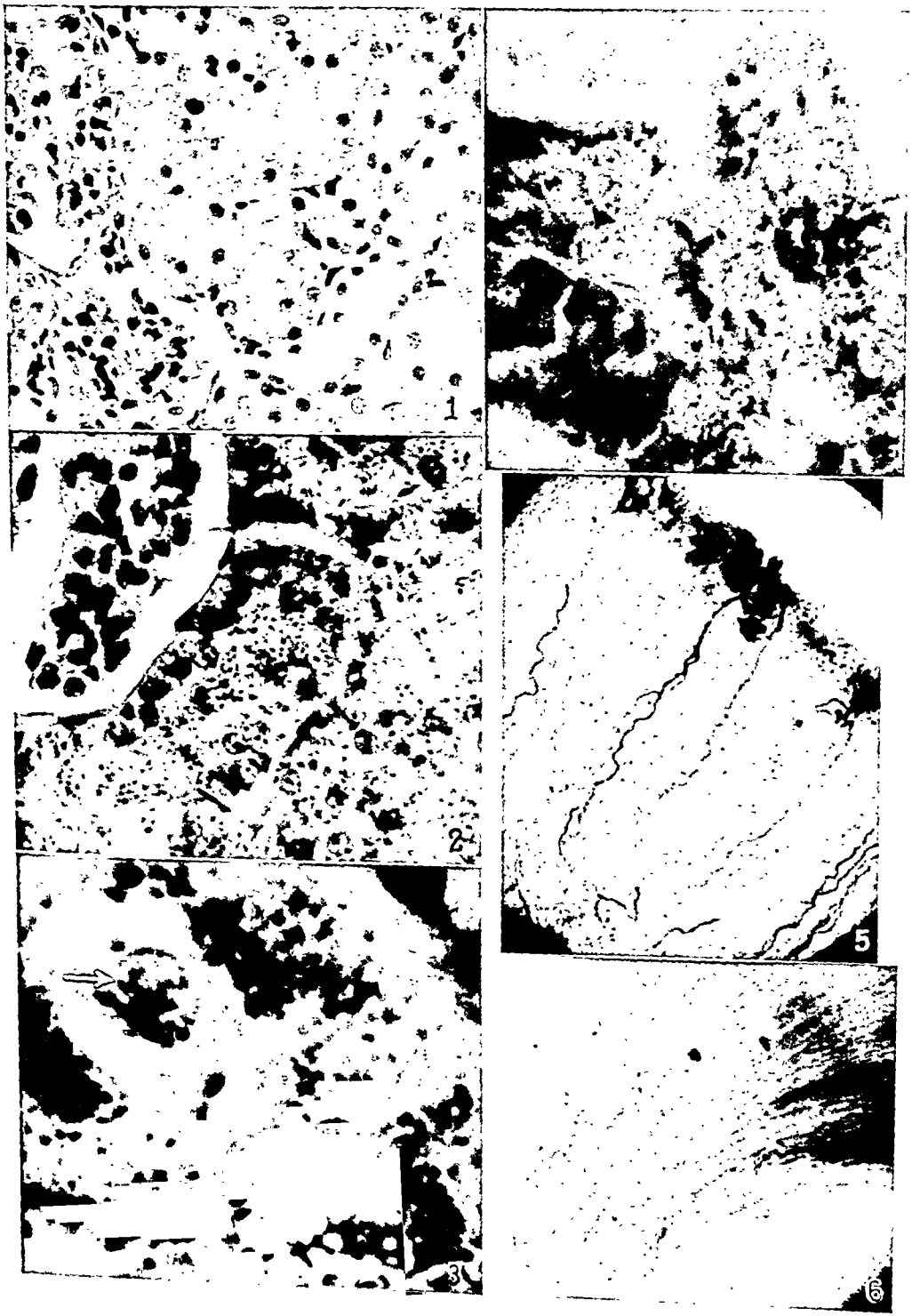
FIG. 2. Experiment 1, 17 hours after injection. Formalin fixation, hemoglobin stain. Many larger intracellular particles of hemoglobin are present. × 892.

FIG. 3. Experiment 1, same slide as in Fig. 2. Arrow points to nucleus of proximal convoluted tubular epithelial cell. The dark particles are intracellular hemoglobin. × 1926.

FIG. 4. Experiment 1, 65 hours after initial injection. Formalin fixation, Berlin blue reaction. Edge of glomerulus shows at top. All of the dark areas represent intracellular hemosiderin in the proximal convoluted tubular epithelium. × 892.

FIG. 5. Experiment 2, 17 hours after initial injection. Formalin fixed, unstained section, 1 mm. thick, cleared by the Spalteholz method. The ascending and descending limbs and some distal convolutions are delineated by intraluminal hemoglobin but the collecting tubules are empty. × 45.

FIG. 6. Experiment 2, 65 hours after initial injection. Formalin fixed, cleared, unstained papilla. A few loops of Henle are visible but the collecting tubules are empty. × 45.



(Rather: Renal athrocytosis)

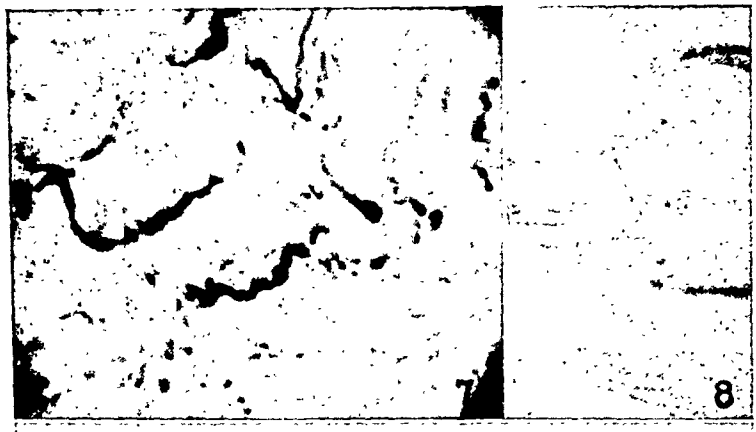
PLATE 4

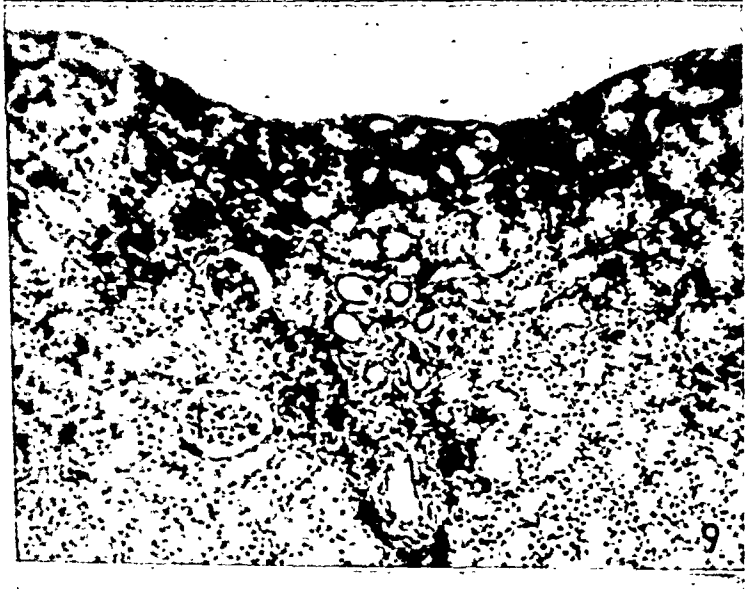
FIG. 7. Experiment 1, 113 hours after initial injection. Formalin fixed, unstained, cleared section, 1 ml. thick. This area in the boundary zone contains some dilated, plugged, tortuous segments. × 46.

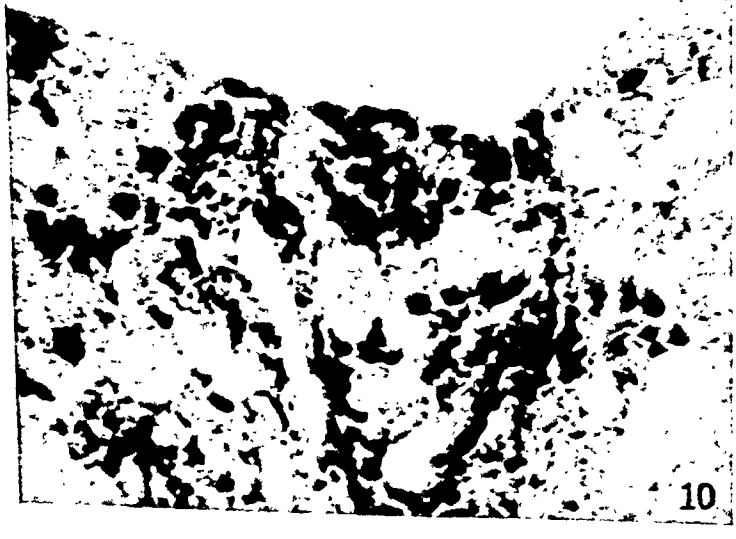
FIG. 8. Experiment 2, 24 hours after initial injection. Formalin fixed, unstained, cleared section, 1 ml. thick. Henle's loops are delineated by intraluminal hemoglobin. × 209.

FIG. 9. Experiment 3, 6 weeks after complete course of hemoglobin injections. Formalin fixation, hematoxylin and eosin stain. A zone of atrophy of tubules and pigment accumulation is shown. × 209.

FIG. 10. Same as Fig. 9, Berlin blue reaction showing that the pigment is hemosiderin.







(Rather: Renal athrocytosis)

PLATE 5

FIG. 11. Experiment 3, 6 weeks after complete course of hemoglobin injections. Formalin fixation, hematoxylin and eosin stain. No evidence of damage. × 100, enlarged 2½ diameters.

FIG. 12. Experiment 2, 42 hours after initial injection. Formalin fixation, hematoxylin and eosin stain. Glomerular space and origin of proximal convoluted tubule are filled with hemoglobin. × 440.

(Rather: Renal athrocytosis)

STUDIES
FROM
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Announcement

Volumes 131 and 132 of the *Studies from the Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

A STUDY OF NERVE PHYSIOLOGY

The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1060 pages with 480 illustrations.

In order that the volumes may be available to those who do not receive them as part of their subscriptions to the *Studies*, extra copies have been printed which are purchasable at the regular price of $2.00 per volume. The two volumes will only be sold together. They can be obtained from the Publication Service, The Rockefeller Institute for Medical Research, York Avenue and 66th Street, New York 21, N. Y., at a price of $4.00, payable in advance.

EFFECT OF ENZYME INHIBITORS AND ACTIVATORS ON THE MULTIPLICATION OF TYPHUS RICKETTSIAE

III. Correlation of Effects of PABA and KCN with Oxygen Consumption in Embryonate Eggs*

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INTRODUCTION

In previous papers of this series (1, 2) it was shown that the growth of typhus rickettsiae in the entodermal cells lining the yolk sac of the egg is inhibited by para-aminobenzoic acid (PABA), by dyes of the toluidin blue group, and by increasing the temperature of incubation from 37.5° to 40.0°C. Potassium cyanide, in doses tolerated by the embryos, was found to neutralize completely the rickettsiostatic action of increased temperature but to have no apparent effect on the action of PABA or toluidin blue.

Potassium cyanide is known to depress cellular respiration while increased environmental temperature and dyes of the toluidin blue group are known to increase the oxygen uptake of many types of tissue. The effect of PABA on the respiration of cells was unknown.

It therefore seemed desirable to make accurate measurements of the oxygen uptake of fertile eggs after injecting these compounds, and to correlate their effects on cellular respiration with their ability to inhibit or accelerate rickettsial growth. The apparatus and technics used for this purpose will be described in detail, since they form a basis for subsequent studies of a similar nature. For the same reason, the statistical analysis of our experimentally determined figures will be given rather fully.

Material and Methods

Methods for infecting fertile chick eggs with typhus rickettsiae, injecting the agents to be tested, and determining the degree of infection were described in detail in Papers I and II of this series.

In the present work oxygen consumption was measured by direct gas analysis. The apparatus to be described was designed to measure the oxygen consumption of eggs in groups of fifteen to twenty, so that the figure obtained in each analysis represents the average utilization

* Aided by a grant from the United States Public Health Service.

of oxygen per egg per hour. Reasons for making analyses of eggs in groups rather than individually will be given below.

Groups of eggs were removed from the incubator and placed in air-tight containers of approximately 7 liters capacity. These containers were buried in a constant temperature water bath for the duration of the analysis. Aluminum desiccators were found to be the most suitable containers for this purpose. The atmosphere within the desiccators was stirred continuously by a propeller mounted on the end of a tuberculin syringe plunger. A portion of the barrel of the syringe served as the bearing. The barrel was inserted into a rubber stopper which in turn was sealed into the lid of the desiccator by means of sealit (Fisher Scientific Co.). Heavy rubber tubing fitted snugly around the external portion of the barrel and plunger prevented water from coming into contact with the bearing and also aided in forming an air

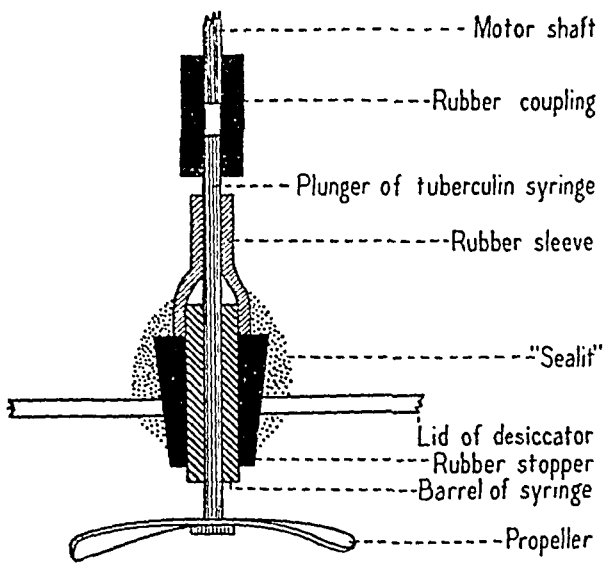

FIG. 1. Details of propeller assembly sealed into lid of desiccator.

tight seal. The end of the plunger projecting to the outside was attached to a motor, mounted on the lid, by means of a rubber coupling (see Fig. 1).

The bearing and shaft were lubricated with glycerine. During the course of the experiment more glycerine was added through the top of the rubber sleeve by injection with a hypodermic syringe. This assembly permitted a pressure differential of 140 mm. of mercury (either positive or negative) while the motor was running at 1750 R.P.M. There was no loss of pressure after several hours. A stop-cock, thermometer, mercury manometer, and serum vial stopper were also sealed into the top of each desiccator.

The water bath designed for these experiments holds four desiccators simultaneously and maintains a temperature of 37.5° ± 0.02°C.

Preliminary experiments (to be discussed below) demonstrated that the accumulation of CO_2 and the increasing humidity during the course of the analysis affected oxygen consumption and viability. Therefore a beaker containing a measured amount of ascarite was placed in the container. This not only removed the CO_2 evolved by the eggs, but also reduced the relative humidity to approximately zero. Neither of these environmental changes appeared to affect the viability of the eggs during their relatively short stay in the respirometer. The use of a dry CO_2 absorbent made it unnecessary to correct for dissolved oxygen.

After placing the eggs to be tested in the container, its ground edges are coated with stop-cock grease and the lid is fastened in place by the use of a 4 inch rubber cuff. One inch of the rubber band encircles the body of the container, the remainder projecting over the lid and thereby sealing it (see Fig. 2). The container is then placed in the water bath. After the air within the containers reaches a temperature of 37.3°C. (as determined by the built-in ther-

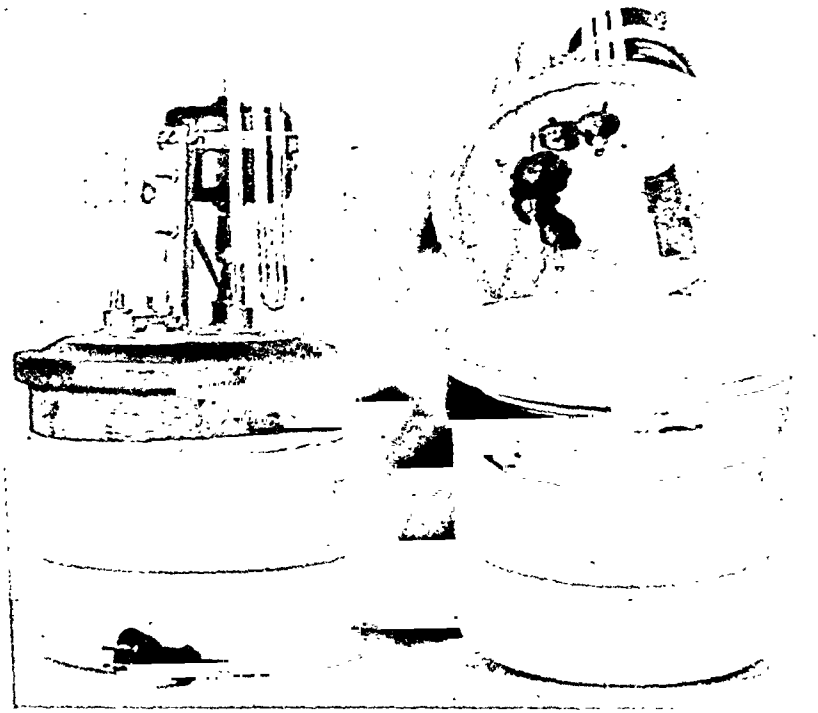

FIG. 2. Details of respirometer chamber to hold twenty eggs, constructed from aluminum desiccator.

mometer) the stop-cock is closed. Five minutes later the first sample of gas is removed. The needle of a 10 cc. syringe, with its plunger closed, is inserted through the serum vial stopper in the lid. The barrel and plunger are coated with heavy stop-cock grease to prevent loss or contamination of the gas sample. An 11 cc. sample of gas is drawn into the syringe. After withdrawal, the needle is quickly removed and the syringe placed in a test tube containing enough mercury to cover the tip of the syringe barrel. A small amount of gas (0.5 cc.) is expelled by depressing the plunger and the assembly is put aside for a half-hour period to permit the entrapped gas sample to reach room temperature. The second sample is

removed when the manometer registers approximately 10 mm. of negative pressure. The elapsed time between samples is noted. The eggs are then removed from the container and candled to determine if any embryos have died. For purposes of calculating oxygen consumption, it is assumed that the occasional embryos which die have lived half of the elapsed time that the eggs were in the respirometer chamber. Oxygen consumption determination is based on the average number of live eggs in the container. When dealing with embryos less than 8 days old, the second sample of gas is removed at the end of 2 hours, regardless of the manometer reading. It is necessary to adopt this arbitrary procedure because of the length of time (6 to 8 hours) required under these conditions for the atmosphere to reach 10 mm. of negative pressure.

The gas samples are analyzed for oxygen by means of a Haldane-Henderson gas analyzer. The results obtained with this apparatus are independent of temperature, barometric pressure, and wet or dry state of the gas sample (3). In order to shorten the time required for an analysis of oxygen consumption oxorbent (Burrel) is used to absorb the oxygen instead of alkaline pyrogallol. Readings which check within 0.002 ml. are obtained in 10 to 15 passes with the former instead of the 30 to 40 required with the latter.

The volume of the eggs, necessary for determining the corrected volume of the respirometer chamber, is found for each setting of eggs by placing a group of them in a 1000 ml. graduate, and pouring water from a volumetric flask (1000 ml.). The amount of water left in the volumetric flask divided by the number of eggs gives the average egg volume.

The volume (in cubic centimeters) of oxygen consumed per egg per hour is calculated from the following:

$$\text{Cc. } O_2\text{/egg/hr.} = \frac{[V_c - (V_E + V_B + V_A)]\left[\frac{R_{CO_2} - R_{O_2}}{S_i} - \frac{R'_{CO_2} - R'_{O_2}}{S'_i}\right]}{\left[\frac{E_1 + E_2}{2}\right](T_2 - T_1)}$$

In certain preliminary experiments in which the amount of CO_2 evolved was measured, the CO_2 absorbent was omitted, and the following formula used for the determination.

$$\text{Cc. } CO_2\text{/egg/hr.} = \frac{[V_c - (V_E + V_B + V_A)]\left[\frac{S'_i - R'_{CO_2}}{S'_i} - \frac{S_i - R_{CO_2}}{S_i}\right]}{\left[\frac{E_1 + E_2}{2}\right](T_2 - T_1)}$$

Where:

V_c = volume of respirometer container;
V_E = total volume eggs;
V_B = volume of beaker holding ascarite;
V_A = volume of ascarite;
R_{CO_2} = volume of gas after absorption of CO_2 (first sample);
R_{O_2} = volume of gas after absorption of O_2 (first sample);
S_i = volume of first gas sample;
R'_{CO_2} = volume of gas after absorption of CO_2 (second sample);
R'_{O_2} = volume of gas after absorption of O_2 (second sample);
S'_i = volume of second gas sample;
E_1 = number of live eggs placed in respirometer;
E_2 = number of live eggs removed from respirometer;
T_1 = time of taking first gas sample;
T_2 = time of taking second gas sample.

oviduct, and egg formation in the hen usually takes from 18 to 30 hours, the development of the blastoderm with differentiation of the three germ layers has already taken place by the time the egg is laid. Thus no two eggs contain embryos of the same age at the time of laying. Another factor is the variable temperature of the environment in which the eggs are maintained before they reach the incubator. In order to average out the above differences in embry-

TABLE II

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Fall Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.89	1.06	0.95	1.39	1.12	0.91
7	1.84	1.42	1.51	1.39	1.52	1.44
9	2.23	2.17	2.68	2.24	2.32	2.61
11	2.99	3.51	3.48	3.20	3.11	3.42
13	8.88	9.01	9.13	9.36	8.91	9.03
15	13.86	13.94	14.36	14.03	14.26	13.92
17	19.50	19.00	19.46	19.56	19.32	19.22

TABLE III

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Winter Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	0.48	0.71	0.34	0.59	0.29	0.57
8	0.82	1.15	1.35	1.01	0.99	0.92
10	2.01	2.48	2.37	2.46	2.17	2.18
12	6.49	6.37	5.98	6.01	6.11	6.36
14	9.87	10.00	9.97	10.39	10.24	10.16
16	13.66	13.41	13.37	13.97	13.82	13.55
18	17.53	17.36	17.74	17.22	17.68	17.34

onic age and development, fifteen to twenty eggs are placed in each respirometer.

Statistical Analysis.—As has been stated the figures obtained in these experiments represent the mean oxygen consumption per egg per hour. It was therefore impossible to employ the statistical methods ordinarily used for comparing populations composed of individuals.

The statistical technique evolved was as follows: Variations in the oxygen consumption between groups of untreated eggs were first found and tabulated. These differences were used as a standard for determining the significance of

differences later found between the control and treated groups of eggs. The eggs used for these preliminary experiments were from the same flock (White Rock variety) and oxygen consumption was measured on settings obtained in the fall, winter, and spring. Each setting was divided into a number of series. Oxygen uptake at different ages was measured simultaneously on groups of twenty eggs from each series. Once used the eggs were discarded. The results are shown in Tables II (fall), III (winter), and IV (spring).

The mean differences were obtained by making all possible combinations of oxygen consumption of embryos of the same age among the several series from

TABLE IV

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Spring Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.42	0.70	0.66	0.91	0.60	0.38
6	1.31	1.62	1.49	1.37	1.78	1.68
7	1.97	1.73	2.20	2.15	1.99	2.41
8	2.50	2.33	1.92	2.47	2.28	2.07
9	2.77	2.99	2.46	2.53	3.01	2.87
10	3.82	4.12	3.67	4.10	3.64	4.02
11	4.99	4.67	4.83	4.91	4.58	4.63
12	5.47	5.96	5.63	5.59	5.45	5.84
13	8.91	8.85	9.22	9.35	9.01	8.97
14	11.22	11.68	11.23	11.74	11.47	11.33
15	13.53	13.68	13.62	13.30	13.78	13.80
16	14.23	14.62	14.54	14.16	14.18	14.49
17	16.07	16.21	16.00	16.51	16.33	16.33
18	17.69	17.55	17.67	17.82	18.03	17.93

each setting of eggs. Thus from Table II, the six oxygen readings for 5 day old embryos give 15 ($6C2$) mean differences; *viz.*, 0.11 (1.06 − 0.95), 0.17 (1.06 − 0.89), 0.06 (1.12 − 1.06), etc. These differences were treated as absolute values; *i.e.*, without regard to sign. The mean of the mean differences (M_{md}) was found from the formula:

$$M_{md} = \frac{\Sigma md}{N}$$

where Σmd = the summation of all possible mean differences of the several series of a given setting of embryos of the same age, and N = the number of mean differences.

In order to compare mean differences it was also necessary to find the standard error of the mean differences (σ_{md}). This was calculated from the following:

$$\sigma_{md} = \sqrt{\frac{\Sigma(M_{md} - md)^2}{N}}$$

Although biological distributions in general follow a cocked-hat form with the normal law as a central type, they frequently show some degree of departure from normality. The type of distribution of oxygen consumption of developing chick embryos was not known. However, it is known that the means of a series of samples drawn from a non-normally distributed parent population will show a close approximation to a normal distribution. From the practical

TABLE V

Mean of Mean Differences and Standard Error of Mean Differences of Oxygen Consumption Determinations in Embryonate Eggs of the White Rock Variety

Age of embryos	Fall setting (Table II)		Winter setting (Table III)		Spring setting (Table IV)	
	M_{md}	σ_{md}	M_{md}	σ_{md}	M_{md}	σ_{md}
days						
5	0.21	0.15			0.24	0.14
6			0.20	0.11	0.23	0.12
7	0.17	0.15			0.28	0.17
8			0.22	0.14	0.28	0.16
9	0.25	0.17			0.29	0.17
10			0.23	0.12	0.26	0.15
11	0.36	0.24			0.20	0.12
12			0.26	0.16	0.23	0.15
13	0.21	0.14			0.23	0.14
14			0.24	0.13	0.27	0.15
15	0.24	0.16			0.22	0.14
16			0.29	0.16	0.24	0.15
17	0.25	0.15			0.23	0.13
18			0.25	0.14	0.21	0.12

point of view in biology, this deviation of the distribution of means and mean differences from the normal form is in general so small as to be of trivial consequence. The form of the above distribution is characterized by the mean of the mean differences (M_{md}) and the standard error of the mean differences (σ_{md}). Table V gives the aforementioned statistics for the several settings.

Differences between the means of mean differences were tested for statistical significance by dividing the difference by the standard error of the difference.

$$\text{The difference in standard units (sigmas)} = \frac{M_{md_1} - M_{md_2}}{\sqrt{\sigma^2_{md_1} + \sigma^2_{md_2}}}$$

This test was made between embryos of the same age but different settings. In no case was there a difference between means of the mean differences that remotely approached significance (3 sigma or greater). Therefore the means

of the mean differences and the standard errors of the mean differences for embryos of the same age but of different settings were combined and weighted to give greater value to the more reliable statistic.

$$\bar{M}_{md} = \frac{N_1 M_{md_1} + N_2 M_{md_2} + \cdots + N_K M_{md_K}}{N_1 + N_2 + \cdots + N_K}$$

$$\bar{\sigma}_{md} = \sqrt{\frac{N_1 \sigma^2_{md_1} + N_2 \sigma^2_{md_2} + \cdots + N_K \sigma^2_{md_K}}{N_1 + N_2 + \cdots + N_K - K}}$$

Where:

$\bar{M}_{md}$ = the weighted mean of the mean differences.

$\bar{\sigma}_{md}$ = the weighted standard error of the mean differences. $N_1 + N_2$ etc. = number of individuals in the several samples.

K = number of samples.

TABLE VI

Statistical Analysis of Figures for Oxygen Consumption of Eggs of the White Rock Variety at Different Embryonic Ages

Age of embryos	Weighted mean of mean differences* $\bar{M}_{md}$	Weighted standard error of mean differences* $\bar{\sigma}_{md}$	Weighted standard error of mean of mean differences* $\bar{\sigma}M_{md}$	Upper value of the five sigma confidence level $\bar{M}_{md} + 5\bar{\sigma}M_{md}$	Minimum differences necessary for significance* $\bar{M}_{md} + 5\bar{\sigma}M_{md} + 3\bar{\sigma}_{md}$
days					
5	0.23	0.148	0.028	0.37	0.82
6	0.22	0.118	0.022	0.33	0.69
7	0.23	0.166	0.030	0.38	0.96
8	0.25	0.155	0.029	0.39	0.87
9	0.27	0.175	0.033	0.43	0.97
10	0.25	0.140	0.026	0.38	0.80
11	0.28	0.195	0.036	0.46	1.06
12	0.25	0.160	0.030	0.40	0.88
13	0.22	0.144	0.027	0.35	0.78
14	0.26	0.145	0.027	0.39	0.84
15	0.23	0.155	0.029	0.37	0.85
16	0.27	0.160	0.030	0.42	0.90
17	0.24	0.145	0.027	0.37	0.82
18	0.23	0.135	0.026	0.36	0.78

* All figures represent cubic centimeters of O_2 consumed per egg per hour.

Table VI gives the foregoing statistics.

As was pointed out previously, the means of a series of samples drawn from a parent population will be approximately normally distributed. Also the mean of the means will approach very closely in magnitude the mean of the parent population. However, when the mean of the parent population is not known, then the best guess as to the true mean is that it will lie somewhere within the range expressed by the mean ± 5 standard errors of the mean. In fact we

would expect the true mean to lie outside of the foregoing range only once in a million times. This is known as the 5 sigma confidence level. Thus in our experiments the expression $\bar{M}_{md} + 5\bar{\sigma}_{M_{md}}$ expresses the upper level of the confidence band and the true mean difference could only be expected to be beyond this value once in a million times. The weighted standard error of the mean of mean differences ($\bar{\sigma}_{M_{md}}$) was found as follows:

$$\bar{\sigma}_{M_{md}} = \frac{\bar{\sigma}_{md}}{\sqrt{N-1}}$$

where N = the number of individuals in the sample. The values of the upper limit of the 5 sigma confidence level are given in Table VI. It was these values which were used in determining the existence of significant or non-significant differences in oxygen consumption between two groups of embryos of the same age.

The following test for significance was used:

$$\text{Mean difference in sigmas} = \frac{\bar{X}_1 - \bar{X}_2 - (\bar{M}_{md} + 5\bar{\sigma}_{M_{md}})}{\bar{\sigma}_{md}}$$

Where:

$\bar{X}_1$ and $\bar{X}_2$ = the figures for oxygen uptake per egg per hour for the two samples of embryos of the same age.

$\bar{M}_{md} + 5\bar{\sigma}_{M_{md}}$ = the upper limit of the 5 sigma confidence band of the mean difference in oxygen consumption to be expected for ebmryos of the same age as the samples.

$\bar{\sigma}_{md}$ = the weighted standard error of the mean difference of oxygen consumption of embryos of the same age as the samples.

Only when the above difference was a positive 3 sigma or greater (expected to occur less than two times in a thousand by chance alone) was the difference between the two samples considered significant.

The figures in the last column of Table VI, obtained by adding three weighted standard errors of the mean differences to the upper value of the 5 sigma confidence level, represent the experimentally determined minimum differences in oxygen consumption considered necessary for significance at each embryonic age.

Preliminary Experiments

Before adopting the above described technique for determining oxygen uptake, certain preliminary experiments were carried out. The method which it was eventually decided to use was chosen as a result of information gained from these experiments.

The Effect of Oxygen and Carbon Dioxide Tensions.—Relatively few observations have been made on the effect of changes in oxygen and carbon dioxide tensions on cellular respiration. In general, however, it has been observed that wide differences in oxygen tension do not influence oxygen consumption.

Slight increases in carbon dioxide tension have been shown to cause a rise in the rate of oxygen uptake, while further increases markedly lower respiration. Since the oxygen consumption of our eggs was measured while they were in closed containers, the effect of CO_2 accumulation required consideration. On the other hand, if the carbon dioxide was absorbed as it was liberated a lowering of the oxygen tension would result. It was therefore necessary that the effect of this factor on oxygen uptake be determined.

Experiment.—A setting of fertile White Rock eggs was divided into two groups. The first group was used to determine the effect of oxygen tension on the oxygen consumption of the developing embryos. The carbon dioxide was removed by placing a beaker of ascarite in the respirometer chamber. This compound also removed water vapor from the atmosphere, reducing the relative humidity to zero.

The second group was used to determine the effect of carbon dioxide tension on oxygen consumption. A beaker containing anhydrone was placed in the container holding the eggs. This compound reduced the relative humidity to zero. In order to prevent the anhydrone from absorbing small amounts of carbon dioxide, it was first saturated with this gas. Excess gas was removed by gentle heating.

As the atmosphere surrounding the eggs was not renewed during the course of an oxygen consumption determination, the carbon dioxide tension varied continuously. The average partial pressure of the gas was calculated from the following:

$$\text{Average partial pressure of } CO_2 = \frac{\text{per cent } CO_2' \cdot P' - \text{per cent } CO_2 \cdot P}{2}$$

Where: P' and P = the barometric pressures at the time of removal of the second and first samples respectively.

The eggs were allowed to remain in the respirometer chambers for a period of 5 hours. An initial gas sample was removed for analysis as soon as temperature equilibrium became established (about 20 minutes after sealing the containers). Thereafter, hourly samples were removed and analyzed.

Discussion.—The hourly differences in oxygen consumption for embryos of a given age which were tested in a carbon dioxide–free atmosphere were statistically insignificant (Table VII). Parnas and Krasinska (7) showed that fertilized eggs of amphibia developed normally in an atmosphere of pure oxygen. Hasselbalch (8), Bohr and Hasselbalch (9), and Krogh (10) in their classical experiments on oxygen consumption of the developing chick embryo, bathed the eggs being tested in a carbon dioxide–free atmosphere and instantly removed any carbon dioxide liberated. They found that the absence of this gas in no way affected either oxygen uptake or carbon dioxide production. It might also be pointed out that the methods of Warburg, Barcroft, Dixon and others used so frequently in the measurement of oxygen consumption of tissues all involve the absorption of carbon dioxide. Thus the absence of carbon dioxide would appear in general to have little effect upon oxygen uptake.

The results shown in Table VII indicate also that the decrease in oxygen concentration resulting from the absorption of CO_2 did not affect the rate of

oxygen uptake. Riddle (11) has reported that for its entire developmental period the embryo presumably requires a concentration of oxygen of not less than 14 per cent. Wesselkin (12) has shown that embryos will live and develop for a limited time in approximately 12 per cent oxygen. Warburg and his coworkers have also shown that the rates of respiration of isolated cells and tissues were independent of oxygen tension within wide limits (13–15). The concentration of oxygen in the respirometer containers in our apparatus was never below 16 per cent. Thus it can reasonably be assumed that the diminished oxygen tension did not affect the respiration of the developing embryos.

The effect of increased carbon dioxide tension on the oxygen consumption of fertile eggs is shown in Table VIII. The 5 day old eggs showed an increase

TABLE VII

Oxygen Consumption of Embryonate Eggs in the Absence of Carbon Dioxide

Age of embryos	Oxygen consumption per egg per hr.				
	1st hour	3rd hour	2nd hour	4th hour	5th hour
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.38	0.32	0.61	0.42	0.29
8	1.35	0.98	1.42	1.01	1.19
10	2.12	2.36	1.99	2.21	2.00
12	5.48	5.61	5.13	5.52	5.50
15	10.64	10.38	10.37	10.49	10.25
17	16.04	16.48	16.33	16.21	16.29

in the rate of oxygen utilization with increased carbon dioxide tension. Although the individual differences were not statistically significant, they probably should be considered significant because of their uniform trend. The 8 and 10 day old embryos showed the same response, and the individual differences were significant. The 12, 15, and 17 day old eggs all showed a significant decrease in oxygen uptake with increasing carbon dioxide tension. The reason for this is not clear.

Root (16, 17) using sea urchin eggs, paramecium, and nerve sections and Burfield (18) using flounder eggs have reported that slight elevations of the carbon dioxide concentration increased oxygen uptake in the foregoing organisms, while large increases in the concentration of the gas greatly lessened oxygen consumption. Romanoff (19) has shown that increased carbon dioxide tension (0.4 per cent) stimulates early growth of the chick embryo. With the advancement of incubation, however, the size of the embryo diminished and at hatching time, many of the embryos were abnormal. Where the atmosphere contained 6.0 per cent carbon dioxide all embryos grew slowly and soon died.

Murray (20) has pointed out that estimations of carbon dioxide production of the developing chick embryo are subject to a number of unknown variables determinable with difficulty. These include variations in the concentration of carbon dioxide in the embryo, albumin and yolk, and contributions made by the carbonates dissolved from the shell.

From the foregoing discussion, it would appear that the most accurate measurement of oxygen consumption of fertile eggs is obtained in a carbon dioxide–free atmosphere.

TABLE VIII

The Effect of Carbon Dioxide Tension on the Oxygen Consumption of Embryonate Eggs

Age of embryos	1st hr.		2nd hr.		3rd hr.		4th hr.		5th hr.	
	V_{O_2}*	P_{CO_2}‡	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}
days	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*
5	0.33	1.18	0.54	1.85	0.57	2.67	0.56	4.59	1.03	5.49
8	0.73	4.01	1.32	5.34	1.35	6.83	1.35	8.84	1.80	10.69
10	2.02	4.68	2.06	4.72	2.37	9.42	2.99	11.30	3.31	13.59
12	5.03	3.78	4.82	6.30	4.58	8.97	4.32	11.95	3.62	14.98
15	10.28	11.72	8.53	15.43	7.61	17.21	5.82	20.10	4.11	21.95
17	16.28	11.20	15.01	20.40	12.32	23.61	10.59	28.40	8.64	34.25

* Cubic centimeters of oxygen consumed per egg per hour.

‡ Average partial pressure of carbon dioxide in millimeters of mercury.

The Effect of Relative Humidity on the Oxygen Consumption of Fertile Eggs.—Romanoff (21) has shown that a definite relationship exists between relative humidity and the growth, calcium metabolism, and mortality of the developing chick embryo. High relative humidity (80 per cent) favored growth and calcium metabolism but caused heavy mortality before hatching. Low relative humidity (40 per cent) retarded growth and calcium metabolism but had no effect on mortality. However, no reports appear to be available concerning the effect of relative humidity on the oxygen uptake of fertile eggs.

Experiment.—A setting of eggs was divided into two groups. One group was placed in a respirometer chamber containing a beaker of ascarite. This resulted in a relative humidity

of 0 per cent and also removed carbon dioxide. The other group was tested in the presence of 30.0 per cent solution of potassium hydroxide. This solution removed the carbon dioxide formed and also produced a relative humidity of 73.8 per cent.

Discussion.—The results obtained are shown in Table IX. There were no significant differences in oxygen uptake for embryos of the same age. Thus the relative humidity appears to have no effect of importance on oxygen consumption. However, it was observed that older embryos displayed a higher mortality with increased humidity, approximately 20 per cent dying within 36 hours after being removed from the respirometer and returned to the incubator. In view of the results of these experiments, ascarite, which absorbed CO_2 and reduced the relative humidity to approximately zero, was adopted in our routine technique.

TABLE IX

The Effect of Relative Humidity on the Oxygen Consumption of Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.	
	High relative humidity (73. 8 per cent)	Low relative humidity (0.0 per cent)
days	*cc.*	*cc.*
5	0.61	0.44
8	1.07	1.37
10	2.36	2.85
12	4.99	5.42
15	10.37	10.42
17	15.81	16.25

The Effect of Rickettsial Infection

Gildenmeister and Haagen (22) in 1940 reported the presence of a toxin, lethal to mice, in the yolk sacs of typhus-infected fertile eggs. Kligler and Oleinik (23), and Olitzki and his coworkers (24) have demonstrated the existence of thermolabile and thermostable toxins in typhus-infected yolk sacs. Bengston, Topping, and Henderson (25) have shown that there is a toxic substance closely associated with rickettsial bodies.

In our method of infecting eggs with typhus rickettsiae, yolk sac material containing unwashed rickettsiae was injected. Thus with each portion of inoculum the eggs presumably received the above mentioned toxins. A series of experiments was carried out to determine the immediate effect of these toxins in the inoculum, as well as the subsequent growth of rickettsiae, on oxygen consumption.

Experiment.—A setting of White Rock eggs was divided into two groups. One group received 0.1 cc. of a 1:50 heavily infected (++++++) suspension of yolk sac material.

The other group served as a control. Oxygen consumption was measured 24 hours before the injections of the yolk sac material, shortly after inoculation (1 hour), and at intervals thereafter. On removal from the respirometer chamber, five of the infected eggs were smeared and stained in order to follow the course of the developing infection.

Discussion.—Table X gives the results obtained in a typical experiment. The oxygen consumption of both series increased steadily from the 6th to the 11th day. The differences between figures for embryos of a given age were not significant. From the 12th day to the 16th day the oxygen uptake of the control group continued to increase in a normal manner. In the typhus-infected group, however, the increase in oxygen uptake with advancing age was at a

TABLE X

The Effect of Infection with Typhus Rickettsiae on Oxygen Consumption of Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.		
	Uninfected eggs (control)	Typhus eggs (infected)	Degree of infection
days	*cc.*	*cc.*	
6	1.23	1.18	0, 0, 0, 0, 0
6.9*			
7	1.56	1.27	0, 0, 0, 0, 0
9	2.08	2.16	0, (+), 0, 0, 0
11	4.65	5.11	0, (+), 0, 0, (+)
12	7.22	5.49	1, 1, (+), 2, 1
13	8.95	6.34	3, 2, 4, 3, 4
14	10.83	7.11	5, 6, 4, 6, 4
16	14.26	2.60	7, 6, 6, 7, 5

* Rickettsiae injected.

much lower rate than in the controls. On the 16th day this group showed a sharp decline in the rate of oxygen uptake. The differences in oxygen consumption between the two groups for embryos of a given age were highly significant.

The presence of the previously discussed toxins in the inoculum appeared to have little effect upon the oxygen consumption of the developing chick embryos from ages 6 to 10 days. However, it was possible that the effect of these toxins was too small to be demonstrable by our technique.

Definite rickettsiae became evident in yolk sac smears on the 12th day and the degree of infection increased steadily, reaching a peak on the 16th day. Thus there appears to be an inverse correlation between the rate of rickettsial growth and the rate of oxygen consumption. The decline in oxygen uptake of the developing embryos appears to be due to the toxins produced by the rickettsiae, as this value declines several days before the degree of infection is great enough to cause the death of large numbers of cells. The low figure obtained for the

16 day old infected embryos may be due to the death of many cells as well as to the effect of rickettsial toxins. At this point, embryonic death was occurring in many eggs.

Although several variables entered into this experiment, it is clear that the presence of rickettsiae in the entodermal cells of the yolk sac, even before fetal death begins to occur, lowers the oxygen consumption of the eggs. The results suggest that the toxins produced by rickettsiae have a depressing effect on cellular respiration, and thus favor the development of the infection. This may be a factor of importance in explaining the favorable effect of antitoxin in typhus.

On careful analysis of the figures obtained in eight experiments (four of which are shown in Tables X, XI, XII, and XIV) it became evident that a slight and temporary rise in oxygen consumption invariably occurred before the more marked and prolonged fall. This effect was noted on the 4th day of infection, at a time when, relatively speaking, very few rickettsiae were present (less than one per high power field). Although the increase in oxygen uptake was on the average only about half as great as the figure which it was decided to regard as statistically significant, its occurrence in all eight experiments makes it clear that it is a real effect. It seems probable then, that the mild concentrations of rickettsial toxins which are present in the early stages of cell invasion have a stimulating effect on cellular respiration. In the higher concentrations which develop rapidly, the effect of these same toxins is apparently a marked reduction in the respiratory rate, and eventual embryonic death.

The Effect of PABA, MABA, and OABA

Nothing was known concerning the effect of PABA, MABA (meta-aminobenzoic acid), and OABA (ortho-aminobenzoic acid) on the respiration of tissues. In the first two papers of this series, we reported that PABA was rickettsiostatic, while MABA and OABA did not influence rickettsial growth. We suggested the possibility that PABA might inhibit rickettsial growth by increasing the metabolic rate of the host cells. The fact that PABA is usually regarded as a member of the B group of vitamins suggested that it might have an effect on intracellular metabolism. It was however possible that all three benzoic acid compounds might affect the metabolism of the host cells in a similar manner and that PABA might act specifically and directly on the metabolism of rickettsiae.

Experiment.—A setting of eggs of the White Rock variety was divided into two series. One series was inoculated with typhus rickettsiae. Each series was further divided into four groups. One group of the non-infected series and one group of the infected series were used as controls. The other groups received 6.6 mg. of PABA, MABA, and OABA respectively. Oxygen determinations were made within 2 hours after the eggs were inoculated with rickettsiae or injected with the aforementioned compounds and at intervals thereafter. Because of the great numbers of eggs required in this experiment it was necessary to use some of the eggs

several times for oxygen determinations. These eggs, however, were never used until they had been in the incubator for several days.

Results.—Table XI shows results typical of those obtained in several experiments. There were no significant differences in oxygen consumption of the developing chick embryos from ages 6 to 11 days. From the 12th day of incubation onward the oxygen uptake of the infected controls and infected egg injected with MABA and OABA was found to decline steadily. These values for embryos of a given age were not significantly different from each other but

TABLE XI

The Effect of PABA, MABA, and OABA on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Oxygen consumption per egg per hr.

Age of embryos	Oxygen consumption per egg per hr.							
	Non-infected series				Infected series			
	Control	6.6 mg. PABA	6.6 mg. MABA	6.6 mg. OABA	Control	6.6 mg. PABA	6.6 mg. MABA	6.6 mg. OABA
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	1.23	1.29	1.68	1.42	1.18	1.27	1.33	1.41
6.9					Eggs inoculated with typhus rickettsiae			
7	1.53	1.53	1.63	1.46	1.38	1.28	1.16	1.49
8.9		PABA injected	MABA injected	OABA injected		PABA injected	MABA injected	OABA injected
9	2.02	2.58	2.42	2.33	1.98	2.14	2.21	2.00
11	4.67	5.50	5.01	4.82	5.17	4.63	5.12	4.75
12	7.20	6.90	7.12	7.35	5.48	6.88	5.92	5.18
13	8.95	12.24	8.86	8.11	6.39	11.35	6.48	6.17
14	10.81	16.00	10.75	9.93	7.14	23.00	7.03	6.57
16	14.21	15.44	13.97	13.03	2.61	18.01	2.37	1.85
18	17.80	16.58	14.87	12.39	—	17.00	—	—

all were significantly lower than the values for non-infected controls of the same age.

The values for oxygen consumption of the non-infected control eggs and non-infected eggs injected with MABA and OABA were not significantly different for 6 to 14 day old embryos. Sixteen day old embryos infected with OABA showed a metabolic rate significantly lower than the controls and those injected with MABA. The values for oxygen consumption of 18 day old embryos injected with MABA and OABA were significantly lower than the values for the controls. Also the metabolic rate of the group injected with OABA was significantly lower than that of the group injected with MABA.

The PABA-treated eggs of both the infected and non-infected series displayed a significant increase in oxygen consumption over the non-infected

controls from the 13th to the 16th days of incubation. The values obtained for 14 and 16 day old PABA-injected eggs of the infected series were significantly greater than those of the non-infected series. Non-infected, PABA-treated, 18 day old embryos were found to have a significantly lower oxygen consumption than the non-infected controls. The values obtained for infected, PABA-treated 18 day old embryos were not significantly different from those found in the non-infected controls.

Discussion.—In summarizing the results of several experiments of this type, it may be said that PABA increased the oxygen consumption of normal eggs, the effect being first seen on the 4th day after its injection, lasting for 4 days and reaching its height at about the middle of this 4 day period. Thereafter, the oxygen consumption fell below that of the untreated eggs. In typhus-infected eggs, PABA affected oxygen consumption in a similar way, the principal difference being that oxygen consumption at its height was much greater than in the uninfected eggs.

This stimulating effect of PABA on respiration is of particular interest, since this finding harmonizes well with previous observations which suggest that rickettsial growth is inversely proportional to the metabolic rate of the host cells (26–28). MABA and OABA, which are not rickettsiostatic, do not increase oxygen consumption; in fact they lower it somewhat. This observation may be explained on the theory that these compounds compete with PABA for linkage in some enzyme system. The detrimental effect of the sulfonamides in rickettsial diseases has been explained in this way.

The fact that PABA raises the oxygen consumption to an even higher level in the presence of rickettsiae than it does in their absence was confirmed repeatedly. The simplest explanation of this observation—and quite possibly the correct one—is that the relatively small numbers of rickettsiae which are present in PABA-treated eggs produce a concentration of rickettsial toxin just sufficient to stimulate cellular respiration. This stimulating effect would persist, since rickettsiae are never completely destroyed, and would be added to the stimulating effect of PABA. The fact that rickettsial infection in untreated eggs causes a slight and temporary increase in oxygen consumption in the early stages of infection has already been brought out.

The Effect of Potassium Cyanide

In our previous work (2) we found that KCN stimulated rickettsial growth. This was presumably the result of a decrease in the metabolic rate in the host cells.

Experiment.—A setting of fertile eggs of the White Rock variety was divided into four groups. One group was untreated and served as a control. The second and fourth groups were inoculated with 0.1 cc. of a suspension of rickettsiae. The third and fourth groups were injected with 10^{-4}M concentration of KCN (based on 20 cc. of yolk).

Results.—Tables XII and XIII show the results typically obtained. The typhus-inoculated eggs gave a curve for oxygen consumption and degree of infection similar to that previously described.

TABLE XII

The Effect of Potassium Cyanide on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.			
	Controls (non-infected)	Typhus-infected	Potassium cyanide	Typhus-infected plus potassium cyanide
days	*cc.*	*cc.*	*cc.*	*cc.*
6	0.37	0.42	0.36	0.36
6.2		Typhus inoculated		Typhus inoculated
7.9			KCN injected	KCN injected
8	1.11	1.20	0.75	0.95
10	2.28	2.46	1.41	1.28
13	4.75	3.48	3.50	1.46
15	8.50	1.63	7.50	
18	22.86		14.28	

TABLE XIII

The Effect of Potassium Cyanide on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.			
	Controls (non-infected)	Typhus-infected	Potassium cyanide	Typhus-infected plus potassium cyanide
days	*cc.*	*cc.*	*cc.*	*cc.*
5	0.38	0.63	0.44	0.97
6		Rickettsiae injected		Rickettsiae injected
7.8			KCN injected	KCN injected
8	1.49	1.99	1.01	0.12
10	3.99	3.29	2.29	2.16
12	5.07	4.05	4.12	2.87
15	10.88	1.68	11.80	
17	14.28		14.71	

The injection of KCN caused an immediate decrease in oxygen uptake. The difference between the control group and the KCN-injected group was not statistically significant. However, this immediate effect has been observed in eight experiments and the difference, therefore, is probably real. Two days after injection of the reagent the oxygen consumption of treated eggs was significantly lower than that of the controls and remained so for the duration of

the experiment (see Table XII). In some experiments the decrease in oxygen uptake was present for several days after the injection of KCN but at later embryonic ages the metabolism of the two groups was not significantly different (see Table XIII).

The fertile eggs inoculated with typhus rickettsiae and subsequently injected with KCN showed essentially the foregoing picture. The decrease in metabolic rate was more pronounced and the embryos died about 24 hours sooner than those inoculated only with rickettsiae. In several experiments the inoculated eggs receiving KCN showed an immediate and significant drop in oxygen consumption. In one experiment (Table XIII) the rate of oxygen consumption in the infected eggs given KCN was only a small fraction of the rate in non-infected and infected untreated controls.

Discussion.—The depressing effect of KCN on the respiration of uninfected eggs is in harmony with known facts concerning the action of this compound. The amount of KCN injected was the largest amount which could be injected without injury to the developing embryos. Quantitatively, the effect was somewhat less than might have been anticipated. It was however, sufficient to explain the enhancement of rickettsial growth which this compound has been observed to cause (2) when injected into eggs in which conditions were unfavorable for rickettsial growth.

In most of our experiments, a single injection maintained the respiratory rate at a lowered level for at least 9 days.

The Effect of PABA Plus KCN

Experiment.—A setting of eggs of the White Rock variety was divided into two groups. Each group was subdivided into four series. One group was inoculated with typhus rickettsiae. One series of each group was used as a control. The second, third, and fourth series of each group received KCN in an amount necessary to give a 10^{-4}M concentration (based on 20 cc. of yolk), 6.6 mg. of PABA, and PABA + KCN respectively. The great numbers of eggs required in this experiment made it necessary to use some of them several times for respiration studies. These eggs were never reused unless they had been in the incubator for several days.

Results.—Table XIV summarizes the results obtained in a typical experiment. The individual effects of rickettsial infection, KCN, and PABA were similar to those already presented.

In the non-infected eggs, PABA caused a striking increase in oxygen consumption. This effect first appeared about 4 days after the injection of the compound, and lasted for 3 days. KCN caused a moderate decrease in oxygen consumption, beginning immediately after injection and lasting for about 9 days. When both PABA and KCN were injected, the stimulating effect of PABA on repiration predominated, so that eggs receiving both compounds showed a marked increase in oxygen consumption as compared with the controls. The rate of oxygen consumption was, however, significantly lower than it was in the eggs receiving PABA alone.

In the infected eggs, the effects of PABA, KCN, and PABA plus KCN were qualitatively similar to the effects of these compounds in the absence of rickettsiae. The stimulating effect of PABA on respiration was, however, quantitatively greater than in the case of the uninfected eggs. KCN caused a decrease in oxygen consumption, the rate even falling significantly below that in the infected controls. When both PABA and KCN were injected, the

TABLE XIV

The Effect of PABA Plus KCN on the Oxygen Consumption of Non-Infected and Typhus-Infected Fertile Eggs

Age of embryos	Oxygen consumption per egg per hr.							
	Non-infected				Typhus-infected			
	Non-infected control	KCN	PABA	PABA plus KCN	Typhus-infected control	KCN	PABA	PABA plus KCN
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	1.35	1.47	1.48	1.17	1.18	1.47	1.37	1.62
6.9					Eggs inoculated with typhus rickettsiae			
7	1.64	1.42	1.74	1.81	1.51	1.58	1.30	1.65
8.9		KCN injected	PABA injected	PABA plus KCN injected		KCN injected	PABA injected	PABA plus KCN injected
9	2.13	1.73	2.70	2.63	2.11	1.95	2.25	1.84
11	4.79	3.92	5.60	5.42	5.28	4.10	4.75	5.09
12	7.33	6.08	6.98	7.01	5.57	4.53	6.99	6.77
13	8.97	7.89	12.36	11.13	6.50	3.72	12.71	11.15
14	11.92	10.78	17.00	15.11	7.63	2.17	22.14	19.32
15	14.32	12.28	16.71	15.45	1.89	—	17.22	16.42
16	16.91	14.58	16.01	16.12	—	—	17.87	17.74
18	18.94	17.99	17.69	17.06	—	—	18.11	18.37

stimulating effect of PABA again predominated, but the respiratory rate was again significantly lower than that of the infected eggs receiving PABA alone.

Effect of Folic Acid

In view of the fact that PABA forms a part of the folic acid molecule, it was decided to test folic acid for a possible effect on rickettsial growth and on cellular respiration in infected and uninfected eggs.

Single injections of 21 mg. of folic acid, an amount corresponding approximately to the optimum dosage of PABA (6.6 mg.), caused a mortality of 80 per cent in the embryos. Injections of half of this amount, however, were well tolerated, and were given on the 2nd, 4th, and 6th days after injecting with

rickettsiae. In other experiments, the eggs received 5 mg. of folic acid daily for 9 days, and in still other experiments 1 mg. and 0.1 mg. daily for 9 days. In none of these experiments did folic acid show any evidence of rickettsiostatic action, or cause any significant increase in oxygen consumption in the uninfected eggs. In one experiment, the larger doses of folic acid appeared to lower oxygen consumption slightly but significantly in the uninfected eggs.

SUMMARY AND CONCLUSIONS

A technique is described for measuring the oxygen uptake of embryonate eggs. Statistical analysis has shown that the method is reliable and accurate. Determinations were made on groups of 15 to 20 eggs, in order to average out individual biological variations. Reduction of the CO_2 tension and relative humidity to approximately zero previous to analysis has been found to be desirable. The oxygen consumption of normal and typhus-infected eggs, untreated and treated with agents previously found to inhibit or enhance rickettsial growth has been studied.

Rickettsial infection caused a slight but significant increase in the rate of oxygen consumption on the 4th day after inoculation, followed by a rapid drop in the rate as the infection developed. The evidence suggests that low concentrations of rickettsial toxins may stimulate respiration, while higher concentrations depress respiration and lead eventually to embryonic death.

PABA, which is rickettsiostatic, markedly increased the oxygen uptake of normal eggs, the effect appearing 4 days after injection and lasting for about 4 days. Thereafter, the rate fell below that of the untreated eggs. In typhus-infected eggs, PABA had similar effects, but the oxygen consumption reached much higher levels. A possible explanation of this fact is suggested. MABA and OABA, which are not rickettsiostatic, did not increase oxygen uptake; in fact they depressed cellular respiration moderately, OABA being more active in this way than MABA. These two compounds may compete with PABA for a position in some respiratory enzyme system.

Potassium cyanide, which enhances rickettsial growth, caused, in concentrations not lethal to the embryos, a moderate drop in the oxygen consumption of normal eggs, the effect starting almost immediately after injection and lasting usually for 9 days. In infected eggs, its effect was more striking. It is probable that rickettsial toxins and KCN act synergistically to depress cellular respiration.

When PABA and KCN were injected simultaneously, the stimulating effect of PABA on respiration predominated. The resulting level of oxygen consumption, though lower than that resulting from PABA alone, was still high enough to inhibit rickettsial growth.

As far as our results go, they support the hypothesis that, within certain limits. rickettsial growth is inversely proportional to the respiratory rate of the

host cells, regardless of the factors which determine that rate. It is not yet clear that PABA owes its rickettsiostatic action to its ability to increase cellular respiration, but this assumption seems reasonable as a working hypothesis. The respiratory mechanism in which PABA participates is not as yet known.

Although PABA forms part of the folic acid molecule, folic acid itself, in concentrations corresponding to effective doses of PABA, did not increase cellular respiration or show rickettsiostatic action.

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STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

I. The Probable Identity of the Enzymes Activated by Chloroform and by Filtrates of Cultures of Beta Hemolytic Streptococci*

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In recent years, interest has been revived in the fibrinolytic properties of human plasma. The rapid lysis of clotted blood has been described in patients with liver disease (1), toxemia of pregnancy (2), shock (3), after typhoid vaccine therapy (4), and pre- and postoperatively (5, 6). The mechanism of fibrinolysis is obscure. Presumably the clot is digested by proteolytic enzymes present in the blood. Any understanding of the problem of clot lysis, therefore, requires an understanding of the proteolytic properties of human plasma. The current study is an investigation of the proteolytic properties of blood, in an attempt to elucidate the mechanism of fibrinolysis.

The proteolytic properties of blood were first noted in 1893 by Dastre (7), who described the digestion of a fibrin clot when it stood in serum for 18 hours. Shortly thereafter, Hedin (8) observed that the blood's proteolytic properties were contained in the euglobulin fraction of plasma which was precipitated with dilute acetic acid. At the same time, Delezenne and Pozerski (9) observed that the addition of chloroform to serum activated a proteolytic enzyme. This chloroform-activated proteolytic property of blood was shown by Tagnon (10) to reside in the acid-insoluble euglobulin fraction, as in the case of the enzymatic activity which was present spontaneously.

Tillett and Garner (11), in 1933 observed that bacteria-free filtrates of cultures of beta hemolytic streptococci contained a water-soluble substance which could not be dialyzed through a semipermeable membrane and which caused rapid lysis of plasma or fibrin clots. This remarkable property was shown by Milstone (12) to depend upon the presence of some substance which he called "lytic factor" in the acid-insoluble euglobulin of plasma. Kaplan (13) and Christensen and MacLeod (14, 15) demonstrated that the same fraction of plasma contained lytic factor and the chloroform-activated proteolytic enzyme. Their studies offered strong evidence that the fibrinolytic properties of streptococcal filtrates were due to the activation of a plasma proteolytic enzyme,

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and that the enzyme so activated was identical with that activated by chloroform.

The present study introduces the use of new quantitative methods for the determination of plasma proteolytic activity, employing the nephelometric estimation of the protein concentration of the enzyme-substrate mixture, as suggested by Grob (16). Using this new method, it has been possible to confirm the probable identity of the plasma proteolytic enzyme activated by chloroform and by filtrates of beta hemolytic streptococci. In the accompanying paper (17) new data will be presented which seem to indicate hitherto undescribed kinetic mechanisms in the activation of plasma proteolytic enzyme.

Methods

Nomenclature (15, 18, 19).—The proteolytic enzyme of plasma or serum has been called plasma (or serum) protease, tryptase, trypsin, fibrinolysin, plasmin, or proteinase. In an effort to prevent confusion, the term plasma proteolytic enzyme will be used throughout. The precursor of this enzyme has been termed plasminogen, tryptogen, or profibrinolysin, but here the term plasma proteolytic enzyme precursor will be used. The enzyme when activated by chloroform will be distinguished as chloroform-activated enzyme. The fibrinolytic principle of cultures of beta hemolytic streptococci was originally called fibrinolysin, and more recently streptokinase. One could speak of the active principle as streptococcal plasma proteolytic enzyme activator. Since this term is cumbersome, we shall use the original name, fibrinolysin, and the enzyme activated by filtrates of cultures of hemolytic streptococci will be termed fibrinolysin-activated enzyme. In this and the paper to follow the term enzyme will be used to mean the crude enzyme preparation used, but does not imply a purified product or even a single substance.

Venous blood was collected under sterile conditions from the antecubital vein of human subjects and transferred immediately to a 50 cc. centrifuge tube containing 0.2 cc. of an oxalate solution for each 5 cc. of blood drawn. The oxalate solution was a mixture of 1.2 gm. of ammonium oxalate and 0.8 gm. of potassium oxalate dissolved in 100 cc. of water (20). The plasma was immediately separated by centrifugation.

Preparation of Enzyme.—The spontaneous proteolytic activity of plasma was observed by Hedin to be due to the presence of some substance in the euglobulin fraction precipitated by dilute acetic acid. Tagnon (10) reported that the same fraction contained the proteolytic enzyme activated by chloroform; and Milstone (12) demonstrated that a similar fraction was necessary for the fibrinolytic action of streptococcal filtrates.

In most of the experiments described in this report, a euglobulin fraction was separated in the following manner. Plasma was added to distilled water in the ratio of 1 cc. of plasma to 19 cc. of water, and the solution was acidified with 1 per cent acetic acid to the pH desired, usually 5.2, as measured with a Beckman laboratory model G pH meter. The resultant precipitate was separated by centrifugation and dissolved in 0.85 per cent sodium chloride solution buffered by M/20 potassium phosphate at pH 7.35 (buffer) usually in one-fourth the original plasma volume. This globulin solution was then activated either by chloroform or by hemolytic streptococcal filtrates.

(*a*) *Chloroform-Activated-Enzyme:* The optimal preparation of chloroform-activated enzyme required meticulous attention to a number of factors. In most experiments, chloroform was added to the globulin solution, and the tube stoppered with a rubber stopper and placed in a water bath at 25°C. for 17 hours. Immediately upon addition of chloroform a heavy white precipitate formed in the globulin solution, usually followed sometime within

an hour by a gray gelatinous clot. This dissolved in about 8 hours, leaving a white granular precipitate. After 17 hours the contents of the tube were transferred to a graduated centrifuge tube and diluted with the buffered saline solution to a suitable volume, usually 10.0 cc. The mixture was then centrifuged for 10 minutes at 2000 R.P.M. in an angle centrifuge (rim diameter = 10 inches). The grayish supernatant solution contained chloroform-activated enzyme.

(*b*) *Fibrinolysin-Activated Enzyme:* Fibrinolysin, the plasma proteolytic enzyme activator in streptococcal filtrates, was prepared from the CO strain of group A, or the H46A strain of group C beta hemolytic streptococcus.[1] Trypticase soy media, Baltimore Biological Laboratories (a tryptic digest of casein, 17 gm., papaic digest of fat-free soy meal, 3 gm., sodium chloride, 5 gm., dipotassium phosphate, 2.5 gm., and dextrose, 2 gm. per liter of media), were inoculated with 1 cc. of an 8 hour broth culture of streptococci for each 100 cc. of media. After 14 hours, 1 N sodium hydroxide was added to the culture at intervals to keep the pH between 7.0 and 7.8 (14). After 24 hours the culture, now profuse, was centrifuged and the supernatant fluid filtered through a Seitz filter. The filtrate, containing streptococcal fibrinolysin in crude form, was used as a source of this material in the experiments described below. However, all experiments were confirmed with a more purified preparation made by dialyzing the broth filtrate in a cellophane casing against frequent changes of distilled water at 4°C. over a period of 3 days, and then concentrating the dialysate by evaporation in the casing to a volume one-fifth of the original.

Fibronolysin-activated enzyme was prepared by the addition of suitable amounts of fibrinolysin-containing filtrates of streptococcal cultures to the buffered globulin solution.

Preparation of Substrate.—In most experiments, the substrate used was an aqueous solution of casein (vitamin-test, Smaco), in a concentration of 0.3 per cent. The casein was dissolved in sufficient 1 N sodium hydroxide to produce a final concentration of 0.004 or 0.006 N, by frequent stirring and gentle heating, never above 56°C. A few drops of toluol were added as a bacteriostatic agent, and the casein solution stored at 4°C.

Measurement of Proteolysis.—Proteolytic activity was measured by adding the solution of substrate to an equal volume of activated enzyme and incubating the mixture in a water bath at 37°C. This temperature was shown by Christensen and MacLeod (15) to be optimal for digestion of casein and gelatin by plasma proteolytic enzyme. In experiments lasting over 3 hours, a few drops of toluol were added as a bacteriostatic agent. The progress of digestion was observed by removing 1 cc. aliquots at suitable intervals and measuring the precipitable protein nephelometrically. This was done with a Coleman, Jr., spectrophotometer at a wave length of 420 mμ, using cuvettes with an internal diameter of 10 mm. To each 1 cc. aliquot, 2 cc. of 12.5 per cent hydrochloric acid was added rapidly, followed by 0.5 cc. of 20 per cent sulfosalicylic acid. To insure uniform turbidity, it was necessary to add the acids as rapidly as possible. Maximal turbidity developed in about 5 minutes. At 420 mμ, the degree of turbidity obeys the Lambert-Beer law to an optical density of approximately 0.650, and concentrations of enzyme and substrate which exceeded this density were avoided. Unless globulin was present, the turbidity decreased rapidly upon addition of sulfosalicylic acid due to the formation of large aggregates. This made it difficult to estimate the concentration of protein quantitatively in the absence of globulin. For the same reason, high concentrations of casein could not be used. The amount of substrate digested was calculated by subtracting the final from the initial optical density of the precipitated protein solutions, and converting the difference in optical density to milligrams of casein. With the instrument and cuvettes used, a solution of casein containing 0.100 mg. per cc. had an

[1] These strains were furnished through the courtesy of Dr. Eleanor Bliss and Dr. L. M. Christensen respectively.

optical density of 0.225. The conversion of differences in optical density to milligrams of casein assumes that none of the globulin is digested during the course of the experiment. Actually in repeated experiments, this was substantially true. At most, the digestion of the activated globulin mixture used accounted for an equivalent of 0.03 to 0.04 mg. of casein.

EXPERIMENTAL

1. The Point of Maximal Precipitation of Chloroform-Activated and Fibrinolysin-Activated Plasma Proteolytic Enzyme.—The point of maximal precipitation of the euglobulin containing the precursor of plasma proteolytic enzyme has not been clearly defined. Tagnon (10) reported that the chloroform-activated proteolytic activity of plasma was present in the euglobulin fraction precipitated by dilute acetic acid at pH 6.0. The euglobulin which Milstone (12) found necessary for the action of fibrinolysin was precipitated by acidifying serum diluted to 20 times its volume in distilled water with 0.32 volumes of acetic acid. This procedure, however, may result in a different pH with each serum. Thus, when 0.5 cc. of 1 per cent acetic acid was added to 2 cc. of plasma diluted with 38 cc. of water, the pH of the resultant mixture varied, in eight experiments, from 5.0 to 5.7. Kaplan (13) and Christensen and MacLeod (14, 15) demonstrated the probable identity of the fractions containing the enzymes activated by chloroform and by fibrinolysin. The following data show that the globulin containing the precursors of chloroform-activated and fibrinolysin-activated enzymes is precipitable from dilute solution maximally at pH 5.2 rather than at pH 6.0.

In a typical experiment, 2 cc. of plasma was added to each of 8 tubes containing 38 cc. of distilled water. Varying quantities of 1 per cent acetic acid were added to each tube so that the pH of the mixture ranged from 6.20 to 4.80. Each tube was then treated in the following manner: The resultant precipitate was separated by centrifugation, the supernatant fluid discarded, and the precipitate dissolved in 1.0 cc. of buffer. Half a cc. of chloroform was then added to each tube, and the mixture incubated at 25°C. for 17 hours. The contents of each tube were then diluted with buffer to a volume of 5 cc. An equal volume of 0.3 per cent casein solution in 0.004 N sodium hydroxide was added, and the mixture incubated at 37°C. for 2 hours. Similarly the globulins precipitated at hydrogen ion concentrations between pH 6.3 and 4.8 were dissolved in 1 cc. of fibrinolysin-containing streptococcal filtrate, and then diluted to 15 cc. with buffer. Five cc. of 0.3 per cent casein was added to a 5 cc. aliquot and the amount of casein digested during 1 hour at 37°C. determined.

As can be seen in Fig. 1, maximal proteolytic activity was present in the globulin precipitated at pH 5.2, whether the enzyme was activated by the fibrinolysin in streptococcal filtrates, or by chloroform. That is, the precursor of the enzyme which was activated by chloroform could not be distinguished from that which was activated by streptococcal fibrinolysin by the use of this technique. Repeated experiments showed that trypticase soy media itself and the streptococcal fibrinolysin in the absence of plasma globulin had no proteolytic activity. Furthermore, the casein solution alone did not hydrolyze measurably when incubated for 24 hours at 37°C.

As one might predict from the variation in the buffering capacity of plasma from person to person, the volume of acetic acid which must be added to diluted plasma to bring the solution to pH 5.2 must be determined separately for each sample.

2. The Optimal pH for the Digestion of Casein by Chloroform-Activated and Fibrinolysin-Activated Plasma Proteolytic Enzyme.—The proteolytic enzyme of plasma is most efficient at the hydrogen ion concentrations likely to be found in blood. Schmitz (21) reported that spontaneously activated plasma proteo-

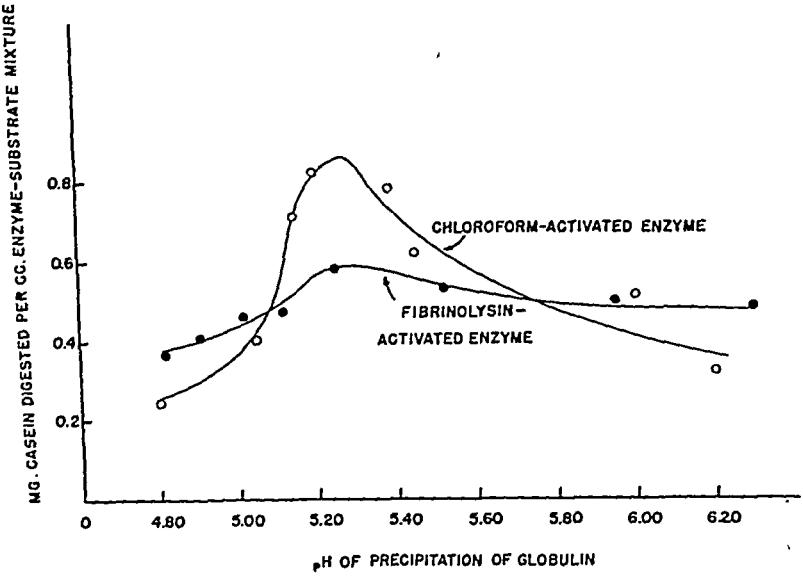

FIG. 1. The point of maximal precipitation of the plasma euglobulin fraction containing proteolytic enzyme precursor as determined by the pH of maximal precipitation. See text.

lytic enzyme digested fibrin most completely when the hydrogen ion concentration of the mixture ranged between pH 7.0 and 8.0. Christensen and MacLeod (15) observed that the proteolytic enzyme of blood activated either by chloroform or by streptococcal fibrinolysin digested gelatin optimally at hydrogen ion concentrations between pH 7.2 and 7.6. The following experiments in which casein was used as a substrate, corroborate these reports.

In a typical experiment, the euglobulin precipitated at pH 5.2 from 2 cc. of pooled plasma was dissolved in 1 cc. of buffer. Half a cc. of chloroform was added and the mixture was incubated for 17 hours at 25°C. The contents of two such tubes were then combined and diluted with buffer to 12 cc. The chloroform and precipitate were then removed by centrifugation. One cc. of the supernatant fluid was placed in each of 11 tubes which contained 4 cc. of M/20 potassium phosphate buffered to various hydrogen ion concentrations between pH 3.3 and 9.7. To each tube was then added an equal volume of 0.3 per cent casein solution in 0.006 N sodium hydroxide. The mixture was then incubated at 37°C. The pH of the

enzyme-substrate mixture was determined with a Beckman pH meter approximately 15 minutes after the mixture was made.

Fibrinolysin-activated enzyme was prepared by dissolving the euglobulin precipitate from 4 cc. of plasma in 2 cc. of streptococcal fibrinolysin, and diluting the solution to 12 cc. with 0.85 per cent sodium chloride buffered at pH 7.35 with potassium phosphate. One cc. was then placed in each of 11 tubes containing 4 cc. of the potassium phosphate buffers. An equal volume of casein was added, the mixture incubated at 37°C., and the pH of the enzyme-substrate mixture measured after 15 minutes.

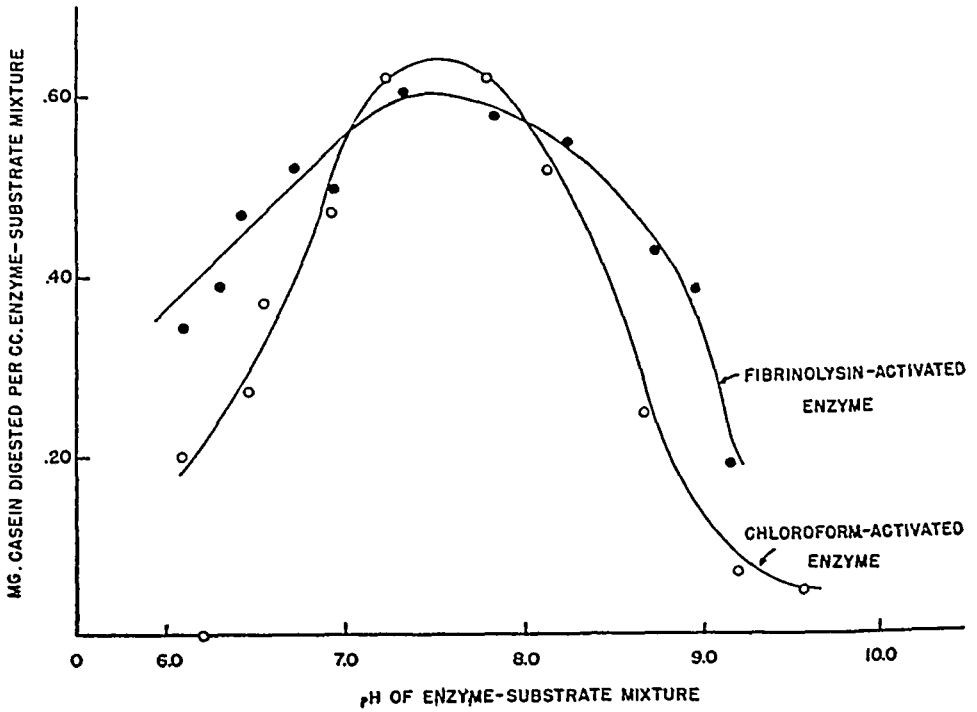

FIG. 2. The optimal pH of the enzyme-substrate mixture for the digestion of casein by plasma proteolytic enzyme, activated by fibrinolysin and by chloroform. See text.

These experiments demonstrated that a crude preparation of plasma proteolytic enzyme, whether activated by chloroform or by streptococcal fibrinolysin digested casein maximally at hydrogen ion concentrations between pH 7.0 and 8.0 (Fig. 2). In all subsequent experiments, therefore, the pH of the enzyme-substrate mixture was kept within these limits. The hydrogen ion concentration of a mixture of an equal volume of casein in 0.004 N or 0.006 N sodium hydroxide and globulin dissolved in pH 7.35 buffer was uniformly between pH 7.5 and 8.0.

3. The Action of Fibrinolysin-Activated and Chloroform-Activated Plasma Proteolytic Enzyme against Various Substrates.—The spontaneously active proteolytic enzyme in plasma was first observed as a result of its ability to digest a fibrin clot (9). This enzyme has also been said to digest casein and gelatin

but not egg albumin (8). When plasma proteolytic enzyme is activated by chloroform, the same substrates are said to be digested (9) and in addition, fibrinogen (22) and hemoglobin (13). Kaplan (13) and Christensen (14) pointed out that the proteolytic enzyme of plasma activated by streptococcal fibrinolysin digested the same substrates. As shown in Table I, the enzyme activated by either method attacked casein and crystalline zinc insulin. With the nephelometric methods used, bovine albumin (fraction V of Cohn), pumpkin seed globulin, egg albumin, lactalbumin, and edestin were not digested by the enzyme activated by either method.

TABLE I

The Digestion of Various Substrates by Plasma Proteolytic Enzyme

Substrate	Concentration of substrate in enzyme-substrate mixture	Amount of substrate digested per cc. of enzyme-substrate mixture in 1 hr.	
		Fibrinolysin-activated enzyme	Chloroform-activated enzyme
Casein	1.5 mg./cc.	0.85 mg.	0.26 mg.
Zinc insulin crystals (Lilly)	40 units/cc.	1.6 units	0.7 units
Bovine albumin (fraction V)	1.25 mg./cc.	0	0
Pumpkin seed globulin	1.0 mg./cc.	0	0
Egg albumin	2.5 mg./cc.	0	0
Lactalbumin	0.25 mg./cc.	0	0
Edestin	0.25 mg./cc.	0	0

No significant difference, then, was noted between the behavior of plasma proteolytic enzyme preparations, activated by chloroform and those activated by streptococcal fibrinolysin, towards various substrates. Repeated experiments, however, showed that the enzyme activated by fibrinolysin was uniformly more active than that activated by chloroform.

4. The Effect of Adding Streptococcal Fibrinolysin to Chloroform-Activated Plasma Proteolytic Enzyme.—The hypothesis that the chloroform-activated and fibrinolysin-activated plasma proteolytic enzymes are identical assumes that the precursors of the two enzymes are identical. Therefore, total activation by either method should remove all the available precursor. Experiments of Christensen (23) showed that this was probably true. The following experiments were designed further to test this hypothesis.

In a typical experiment, globulin was prepared from 4 cc. of plasma. The fibrinolysin-activated proteolytic activity was determined on exactly one-third of this globulin solution by adding 0.1 or 0.2 cc. of streptococcal fibrinolysin, amounts much less than necessary to activate all the precursor present. The remaining globulin was treated with chloroform for 17 hours in the usual manner. At the end of that time, the enzymatic activity was determined on exactly half the specimen. To the other half, fibrinolysin was added in the same

TABLE II

Digestion of Casein by Fibrinolysin-Activated Enzyme, Chloroform-Activated Enzyme, and Enzyme Activated by Chloroform and Fibrinolysin in Succession

Preparation	Enzyme activated by fibrinolysin (A)	Enzyme activated by chloroform (B)	Calculated sum of A and B	Enzyme activated by chloroform followed by fibrinolysis
	*mg./cc.**	*mg./cc.**	*mg./cc.**	*mg./cc.**
11–21–46	0.34	0.37	0.71	0.81
11–25–46	0.74	0.23	0.97	0.95
1– 4–47	0.59	0.26	0.85	0.89
1– 8–47	0.74	0.09	0.83	0.89
2– 7–47	0.44	0.17	0.61	0.75
2–11–47	0.37	0.30	0.67	0.70
2–13–47	0.11	0.20	0.33	0.43

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture.

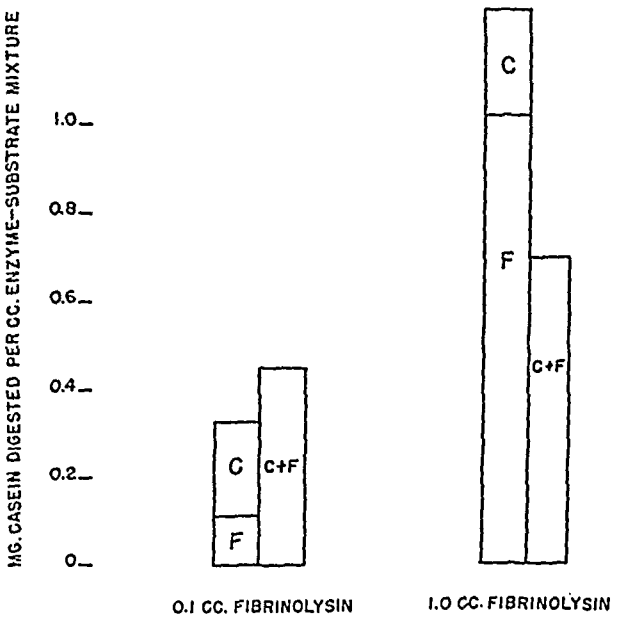

FIG. 3. The activation of plasma proteolytic enzyme by chloroform, by streptococcal fibrinolysin, and by combinations of the two. This graph illustrates the proteolytic activity resulting from treatment of a globulin preparation with chloroform (*C*) and with fibrinolysin (*F*). This is compared with the enzymatic activity of the globulin preparation treated successively with the same amounts of chloroform and fibrinolysin (*C* + *F*). With small amounts of fibrinolysin, the enzymatic activity evoked by the combination was roughly the sum of the activities of the globulin treated with chloroform or fibrinolysin alone. With large amounts of fibrinolysin the activity evoked by chloroform followed by fibrinolysin was less than that evoked by fibrinolysin alone.

concentrations as before, and the resultant activity measured. The concentration of globulin was the same in all preparations in the final enzyme-substrate mixture.

These experiments demonstrated that the enzymatic activities induced by chloroform and by small amounts of fibrinolysin were approximately additive (Table II). In most preparations the activity resulting from successive treatment with the two agents was actually a little greater than the sum of the separate enzymatic activities. This may be because treatment of globulin with chloroform retards the deterioration of fibrinolysin-activated enzyme, promotes increased activation of enzyme by streptococcal fibrinolysin, or both. These possibilities are discussed in the accompanying paper (17).

Christensen (14) observed that the addition of concentrated fibrinolysin to chloroform-activated plasma proteolytic enzyme resulted in proteolytic activity less than that produced by fibrinolysin alone. Similarly in the experiments reported here, when fibrinolysin alone was used in quantities sufficient to activate all available plasma proteolytic enzyme, more casein was digested than was the case when the same amount of fibrinolysin was added to the same amount of globulin, previously activated by chloroform. That is, the enzymatic activities evoked by chloroform and fibrinolysin were no longer additive.

The results of such an experiment are represented graphically in Fig. 3. The proteolytic activity induced by chloroform followed by a small amount of fibrinolysin was somewhat greater than the sum of the activities of a preparation activated by chloroform and another activated by the same amount of fibrinolysin. The enzymatic activity induced by chloroform followed by a large amount of fibrinolysin, on the other hand, was actually less than that produced by the same amount of fibrinolysin alone. A possible explanation of this finding will be discussed below.

DISCUSSION

The data presented support the view that the plasma proteolytic enzymes activated by streptococcal fibrinolysin and by chloroform are identical. This conclusion is suggested by experiments which show that the precursor of the enzyme activated in either manner is present in the same acid-insoluble euglobulin; that the enzyme activated in either manner digests casein optimally at hydrogen ion concentrations between pH 7.0 and 8.0; that the same substrates are digested; and that the enzyme can be activated successively by both agents, but only until the available precursor is exhausted.

A crude separation of the enzyme was made by acid precipitation. The precursor of plasma proteolytic enzyme was present in the euglobulin fraction precipitated at pH 5.2. This fraction is, of course, a mixture of many substances, and includes such proteins as fibrinogen and prothrombin, as well as the precursor of plasma proteolytic enzyme.

The optimal hydrogen ion concentration for the digestion of casein by plasma proteolytic enzyme is between pH 7.0 and 8.0. This hydrogen ion concentra-

tion approximates that previously described by Schmitz (21) and Christensen and MacLeod (15) for the digestion of fibrin and gelatin. Trypsin similarly acts optimally at an approximately neutral pH. Earlier workers described the plasma proteolytic enzyme as "plasma trypsin," but the experiments of Kaplan (24) and Christensen and MacLeod (15) clearly demonstrate that the plasma proteolytic enzyme and trypsin are not identical.

The substrates digested by plasma proteolytic enzyme include fibrinogen, fibrin, gelatin, hemoglobin, casein, and crystalline zinc insulin. Prothrombin too is apparently destroyed by plasma proteolytic enzyme, though the evidence for this is inferential (22). No difference was observed in the substrates attacked by the enzyme activated in either manner.

When small amounts of streptococcal fibrinolysin were added to plasma proteolytic enzyme already activated by chloroform, the preparation increased in activity. The increase in activity was approximately equal to the proteolytic activity induced by fibrinolysin acting alone on the same euglobulin preparation. That is, the activities induced by chloroform and by small amounts of fibrinolysin were additive. When fibrinolysin was used in concentrations sufficient to activate plasma proteolytic enzyme maximally, the proteolytic activities of chloroform-activated and fibrinolysin-activated enzymes were no longer additive. In fact, the addition of such an amount of fibrinolysin to chloroform-activated enzyme actually resulted in less activity than that produced by the same amount of fibrinolysin alone. These experiments imply that the precursors of fibrinolysin-activated and chloroform-activated enzymes are identical or, less likely, that some substance in plasma is necessary for the activation of each. The precursor may be activated by chloroform, but during the period of observation all the precursor cannot be so activated. The remaining precursor may be activated by streptococcal fibrinolysin. Therefore, maximal amounts of fibrinolysin added to globulin solution previously activated by chloroform cannot activate more precursor than can be activated by the same amount of fibrinolysin alone.

In the experiments described, large amounts of fibrinolysin added to chloroform-activated enzyme actually evoked less enzymatic activity than that evoked by the same amount of fibrinolysin alone. This may be because chloroform may not only activate precursor, but also denature some of the precursor or enzyme as well (23). Another mechanism, however, is possible. Perhaps some of the chloroform-activated enzyme had already deteriorated by the time fibrinolysin was added. Once deteriorated, the enzyme may be incapable of reactivation by fibrinolysin. Experiments with fibrinolysin-activated enzyme lend force to this suggestion. In the accompanying paper, it will be shown that fresh fibrinolysin did not reactivate fibrinolysin-activated enzyme once this had deteriorated (17).

SUMMARY

1. The experiments reported suggest that the plasma proteolytic enzyme activated by streptococcal fibrinolysin is identical with that activated by chloroform.

2. The precursor of this plasma proteolytic enzyme is precipitated with the euglobulin fraction of plasma at pH 5.2.

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STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

II. Some Factors Influencing the Enzymes Activated by Chloroform and by Streptococcal Fibrinolysin*

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Mammalian plasma contains a proteolytic enzyme in inactive form. Early studies indicated that in shed blood the enzyme gradually became activated spontaneously, and the blood acquired proteolytic properties (1, 2). When the plasma was treated with a variety of substances, including chloroform or other organic solvents (3) its proteolytic activity was greatly increased, although the activation proceeded slowly. Filtrates of cultures of beta hemolytic streptococci also increased the proteolytic activity of plasma (4), apparently by activating the same proteolytic enzyme as chloroform (2, 5, 6). Activation of plasma proteolytic enzyme with streptococcal fibrinolysin, however, is dramatic and sudden, suggesting that the mechanism of action of chloroform and streptococcal fibrinolysin differ.

In order to delineate the conditions necessary for the quantitative determination of plasma proteolytic enzyme, a study has been made of the activation of the enzyme by chloroform and by streptococcal filtrates. This study indicates that activation of plasma proteolytic enzyme by chloroform is incomplete and slow, requiring at least 16 hours for the development of maximal activity. On the other hand, activation with maximal amounts of streptococcal fibrinolysin occurs almost instantly.

Earlier reports (5, 7) suggested that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin is a catalytic process. Data will be presented in this paper which indicate that in the presence of some substance altered by chloroform or heat, the activation of plasma proteolytic enzyme by streptococcal fibrinolysin behaved as if it involved a stoichiometric reaction. Streptococcal fibrinolysin seemed to react in molecular proportions with a substance in plasma euglobulin which limited the activation of plasma proteolytic enzyme. These data reopen the question as to the mode of activation of plasma proteolytic enzyme by streptococcal fibrinolysin.

The quantitative estimation of enzymatic activity requires a knowledge of the influence of changes in concentration of enzyme and substrate upon the course of digestion. Earlier studies (8) indicated that the digestion of gelatin

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by plasma proteolytic enzyme was proportional to the square root of the concentration of enzyme, and to the square root of the duration of digestion. That is, plasma proteolytic enzyme was said to obey Schütz's law.

The rate of digestion by plasma proteolytic enzyme has been reinvestigated. These studies suggest that like other hydrolytic enzymes, plasma proteolytic enzyme digests casein in direct proportion to the concentration of the enzyme and to time, at least during the early course of digestion. Furthermore, it has been shown that the plasma proteolytic enzyme preparation, when activated by filtrates of cultures of beta hemolytic streptococci, deteriorates after incubation for approximately 1 hour; but the same enzyme activated by chloroform does not deteriorate for several hours. These studies indicate that chloroform may remove some substance from plasma which permits plasma proteolytic enzyme to deteriorate.

Methods

The nomenclature and all the methods employed in the following experiments have been described in the preceeding paper (6).

EXPERIMENTAL

I. The Kinetics of Activation of a Plasma Proteolytic Enzyme by Chloroform

1. The Optimal Duration and Temperature of Incubation of Plasma Globulin and Chloroform in the Activation of Plasma Proteolytic Enzyme.—The quantitative determination of the amount of chloroform-activated plasma proteolytic enzyme requires a knowledge of the optimal duration of incubation of the chloroform-globulin mixture, and the optimal temperature of this activation. When chloroform is added to whole plasma, the proteolytic activity of the mixture slowly increases, reaches a maximum in 7 to 10 days, and then decreases (3, 9). The following experiments indicate that the peak of proteolytic activity is reached in a much shorter time if the precursor of plasma proteolytic enzyme is first separated from the rest of the plasma by precipitation with the euglobulin fraction at pH 5.2.

In order to determine the optimal time of activation of plasma proteolytic enzyme with chloroform, equal amounts of chloroform were added to aliquots of a solution of plasma euglobulin in buffer, and the resultant mixtures then incubated. The course of activation was determined by measuring the proteolytic activity of aliquots which were incubated for increasingly long periods of time.

In a typical experiment, 20 cc. of plasma was added to 380 cc. of distilled water, and sufficient 1 per cent acetic acid was added to bring the hydrogen ion concentration to pH 5.2. The resultant precipitate was separated by centrifugation and dissolved in 22 cc. of buffer. One cc. portions of the globulin solution were pipetted into each of 21 large test tubes, and half a cc. of chloroform was added to each. The tubes were then stoppered and shaken for

10 seconds. Ten tubes were placed in a water bath at 25°C. and 10 in a bath at 37°C. The proteolytic activity of the twenty-first mixture was determined immediately. After appropriate intervals, the activity of the other mixtures at each temperature was measured. To determine proteolytic activity, the contents of each tube were transferred to a graduated centrifuge tube and the aqueous phase diluted with buffer to 5 cc. The chloroform and precipitate were separated by centrifugation. Two cc. of 0.3 per cent casein in 0.004 N sodium hydroxide was then added to a 2 cc. aliquot of chloroform-activated enzyme, and the amount of casein digested in 1 hour at 37°C. was determined nephelometrically.

As can be seen in Fig. 1 short periods of incubation of the chloroform-globulin mixture were unsatisfactory, because the amount of proteolytic activity present

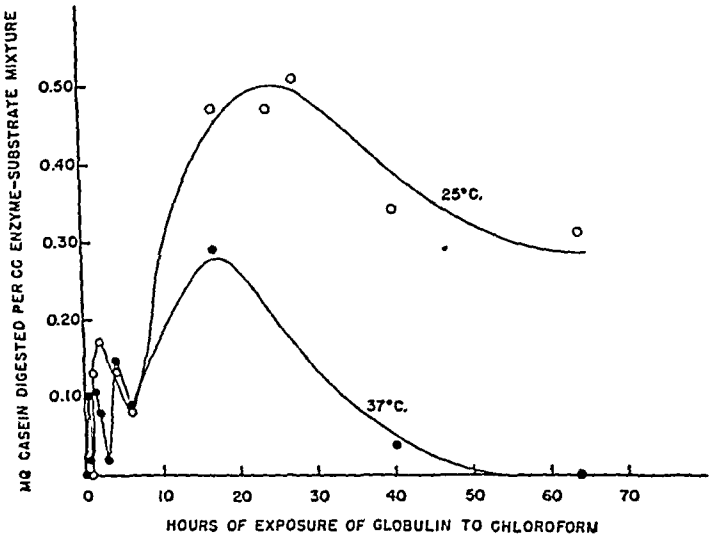

FIG. 1. The influence of the duration of incubation of chloroform-globulin mixtures on plasma proteolytic activity at 25° and 37°C. See text.

was extremely variable. This may have been due to the formation of a pale gray clot which could be broken up only with difficulty. After about 8 hours of incubation the clot lysed and thereafter consistent results were obtainable. The peak of activity was reached between 16 and 28 hours, after which activity declined.

Incubation of the chloroform-globulin mixture at 25°C. resulted in much greater proteolytic activity than incubation at 37°C. No measurable proteolytic activity was present after incubation of the mixture at 4° or 56°C. for 17 hours. These experiments indicate that incubation of a mixture of plasma euglobulin solution and chloroform for 16 to 28 hours at 25°C. was the most effective of the combinations of time and temperature tested in the activation of plasma proteolytic enzyme.

2. The Optimal Values of Buffer and Chloroform Necessary for the Maximal Activation of the Proteolytic Enzyme in 2 cc. of Plasma.—The quantity of buffer in which the euglobulin precipitate of plasma was dissolved had a material effect upon the proteolytic activity which appeared upon incubation with chloroform. If the globulin precipitate was dissolved in more than 2 cc. of buffer before the addition of chloroform, very little active enzyme was found. Table I tabulates the results of an experiment designed to demonstrate which volume of buffer, used to dissolve the euglobulin precipitate of 2 cc. of plasma,

TABLE I

Influence on Proteolytic Activity Evoked by 0.5 Cc. of Chloroform of the Volume of Buffer Used to Dissolve the Euglobulin Precipitate of 2 Cc. of Plasma

Volume of buffer	Enzymatic activity
cc.	*mg./cc.**
0.5	0.70
1.0	0.68
2.0	0.44

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

TABLE II

Influence on Proteolytic Activity of the Volume of Chloroform Added to the Euglobulin Precipitate of 2 Cc. of Plasma Dissolved in 0.5 Cc. of Buffer

Volume of chloroform	Enzymatic activity
cc.	*mg./cc.**
0.1	0.43
0.3	0.43
0.5	0.46
1.0	0.42

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

resulted in greatest enzymatic activity. As can be seen, the maximal proteolytic activity observed was in the tubes in which the globulin precipitate of 2 cc. of plasma was dissolved in 0.5 cc. of buffer. This volume was used in most experiments, although in some the globulin was dissolved in 1.0 cc. of buffer.

The volume of chloroform added to the buffered globulin solution was of importance, although this was minimized by thorough mixing before incubation. In experiments in which chloroform and globulin were mixed thoroughly, a volume of 0.5 cc. of chloroform evoked the most proteolytic activity when the globulin was dissolved in a volume of 0.5 cc. or 1.0 cc. (Table II). In some experiments in which mixing was not thorough, a volume of chloroform of 0.2 cc. or less induced much less activity than 0.5 cc. Furthermore, repeated experiments indicated that a volume of chloroform of 1.0 cc. resulted in less

activity than 0.5 cc., perhaps because the larger volume of chloroform denatured more of the enzyme.

In summary, the proteolytic activity evoked by chloroform was maximal under the conditions studied when the euglobulin precipitate of 2 cc. of plasma was dissolved in 0.5 cc. of buffer, mixed thoroughly with 0.5 cc.o f chloroform, and incubated for 16 to 28 hours at 25°C.

II. The Kinetics of Activation of Plasma Proteolytic Enzyme by Streptococcal Fibrinolysin

1. The Influence of Increasing Concentrations of Streptococcal Fibrinolysin upon the Immediate Proteolytic Activity of Plasma Globulin.—In order to determine the amount of proteolytic enzyme in plasma which can be activated by streptococcal fibrinolysin, the effect of increasing concentrations of streptococcal fibrinolysin upon proteolytic activity was studied.

In a typical experiment, the euglobulin precipitate of 16 cc. of pooled plasma was dissolved in 15 cc. of buffer. Varying volumes of fibrinolysin prepared from the H46A strain of beta hemolytic streptococcus were then added to 1 cc. aliquots of the globulin solution, and the volume in each tube brought to 5 cc. with buffer. One cc. aliquots of these mixtures were immediately diluted with 1 cc. of buffer, and 2 cc. of 0.3 per cent casein in 0.006 N sodium hydroxide was added. The progress of digestion during 1 hour at 37°C. was measured.

As may be seen in the upper curve of Fig. 2, the proteolytic activity of the globulin solution increased as the concentration of fibrinolysin increased, until a maximum was reached.

2. The Stability of Streptococcal Fibrinolysin-Activated Enzyme.—Christensen (5) demonstrated that the plasma proteolytic enzyme activated by small amounts of fibrinolysin did not reach the peak of its activity for several hours. The experiments to be described suggest, on the contrary, that prolonged incubation of small amounts of fibrinolysin with a solution of globulin which has otherwise been untreated does not increase proteolytic activity. Indeed, the activity of globulin activated by fibrinolysin began to decrease within the 2nd hour, regardless of the concentration of fibrinolysin.

In the experiment described in the preceeding section, 1 cc. aliquots of the globulin-fibrinolysin mixtures were incubated for 4 hours at 37°C. before the addition of casein, and the proteolytic activity of the mixture was then measured as before. The proteolytic activity of another set of 1 cc. portions of the same globulin-fibrinolysin mixtures was then measured after incubation for 3½ hours at 25°C.

As can be seen in Fig. 2, the fibrinolysin-induced proteolytic activity of globulin had decreased after incubation for 3½ hours. The decrease was apparently greater at 37°C. than at 25°C., and as marked with low as with high concentrations of fibrinolysin. That is, after the proteolytic enzyme in plasma euglobulin had been activated by streptococcal fibrinolysin, its activity decreased rapidly.

3. The Interrelation of the Concentrations of Streptococcal Fibrinolysin and Plasma Globulin, and Plasma Proteolytic Activity.—The activation of plasma proteolytic enzyme by streptococcal filtrates has been said to be catalytic in nature (5, 7). The hyperbolic relationship between fibrinolysin concentration and proteolytic activity described above might be explained otherwise, namely, that streptococcal filtrates may activate plasma proteolytic enzyme by reaction with some substance in plasma in accordance with the law of mass action. If this were true, then the amount of proteolytic activity evoked by mixtures of

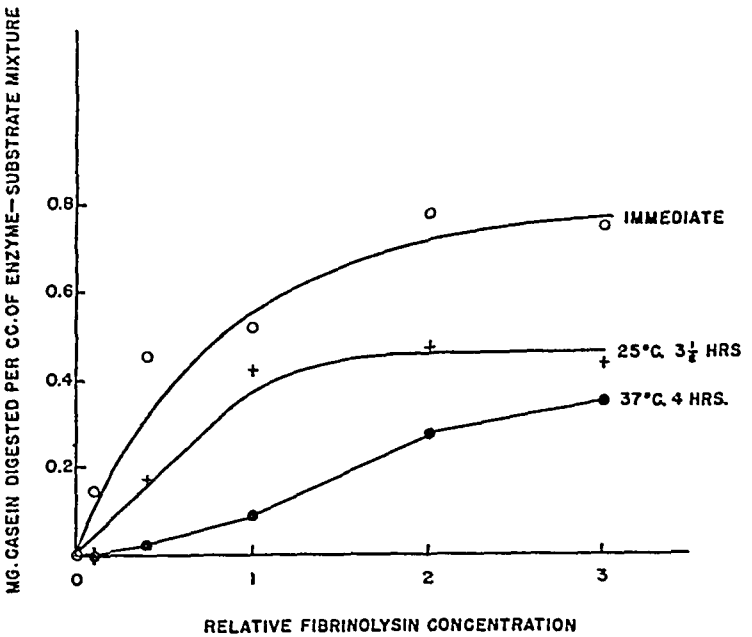

FIG. 2. The influence of increasing concentrations of streptococcal fibrinolysin on the proteolytic activity of plasma globulin solution, and the effect of incubation on the activated emzyme. Proteolytic activity is plotted against the amount of fibrinolysin added to globulin solution. The upper curve represents the proteolytic activity immediately after the addition of fibrinolysin; and the middle and lower curves, the proteolytic activity after the fibrinolysin-globulin mixtures had been allowed to stand at 25 and 37°C. for 3½ and 4 hours respectively.

streptococcal filtrates and of globulin should depend upon the product of their respective concentrations. The following experiment was designed to test this hypothesis.

A series of dilutions of streptococcal fibrinolysin was prepared, using buffer as a diluent. Similarly, plasma globulin was prepared in two dilutions, using buffer as a diluent. Equal volumes of each dilution of streptococcal fibrinolysin and of each dilution of globulin were mixed. This resulted in a series of tubes in which the fibrinolysin and globulin concentrations varied, but the products of the concentrations of fibrinolysin and globulin coincided at various points. To each mixture an equal volume of 0.3 per cent casein was added, and the enzyme-substrate mixture was then incubated at 37°C. for 1 hour.

The data from one experiment such as this have been plotted in Fig. 3. The ordinate represents the enzymatic activity of each preparation, expressed in milligrams of casein digested during the 1 hour period. The abscissa represents the product of the concentrations of fibrinolysin and globulin, expressed arbitrarily. As can be seen, the effect on enzymatic activity of doubling the concentration of fibrinolysin is the same as the effect of doubling the concentration of globulin within the limits tested. These results are compatible with a stoichiometric reaction.

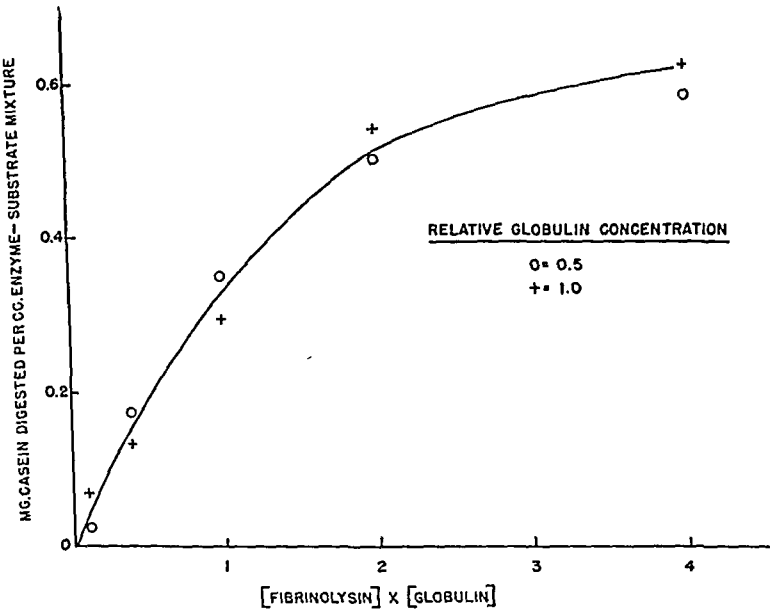

FIG. 3. The relationship between the product of the concentrations of fibrinolysin and globulin solutions and plasma proteolytic activity. The concentrations of fibrinolysin and globulin were varied independently and the resultant proteolytic activity was plotted against the product of the concentrations of fibrinolysin and globulin. See text.

4. The Effect of Fresh Fibrinolysin on Deteriorated Fibrinolysin-Activated Enzyme.—The experiments just described suggested that when streptococcal fibrinolysin activates plasma proteolytic enzyme, the former reacts with some substance in euglobulin in molecular proportions. If this is true, then the quantity of inactive precursor which remains after activation should be inversely proportional to the amount of fibrinolysin added originally. The following experiments were designed to test this possibility.

Fibrinolysin was added in increasing amounts to aliquots of plasma globulin dissolved in buffer. Each mixture was divided into three parts. The first part was assayed for proteo-

lytic activity immediately. The other two parts were allowed to stand in a water bath at 37°C. for 4 hours. Then the enzymatic activity of one of these aliquots was measured; and at this time, 1 cc. of streptococcal fibrinolysin was added to each of the tubes of the third group, and the resultant enzymatic activity assayed immediately.

When proteolytic activity was measured immediately, the activity of the globulin preparation varied directly with the amount of fibrinolysin added until a maximum was reached (Fig. 4). The larger amounts of fibrinolysin seemed to saturate the substance in globulin with which it reacted. After 4 hours, the

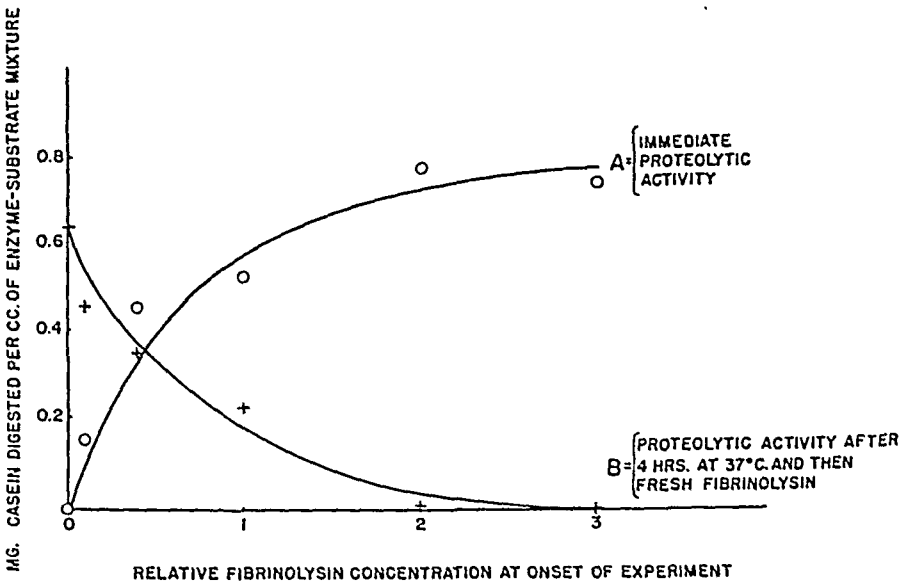

FIG. 4. The effect of additional fibrinolysin on plasma proteolytic enzyme previously activated by fibrinolysin. Varying amounts of fibrinolysin were added to aliquots of globulin solution, and the proteolytic activity measured immediately (curve A). These mixtures were allowed to stand at 37°C. After 4 hours the proteolytic activity of an aliquot of each mixture was again determined. At the same time, 1 cc. of fibrinolysin was added to another aliquot of each mixture and the resultant proteolytic activity was measured. Curve B represents the net proteolytic activity attributable to the addition of fresh fibrinolysin.

enzymatic activity of these preparations decreased considerably, particularly in those tubes to which the smaller amounts of fibrinolysin had been added. When 1 cc. of fibrinolysin was added to each tube after the 4 hour period, there was a great increase in activity in those tubes which originally contained the least fibrinolysin, and no increase in activity in those originally saturated with fibrinolysin. These data, with those described in the preceding section, demonstrate that in the activation of plasma proteolytic enzyme, streptococcal fibrinolysin reacts stoichiometrically with some substance in the globulin preparation.

5. The Effect of Heat or Chloroform on the Activation of Plasma Proteolytic Enzyme by Streptococcal Fibrinolysin.—Christensen and MacLeod (5, 7) re-

ported that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin was catalytic in nature, and in the absence of inhibitory substances the kinetics of activation appeared to be that of a first order unimolecular reaction. A small amount of fibrinolysin was as effective as larger amounts, although the time necessary for complete activation was longer. In the experiments described in the preceding sections, fibrinolysin appeared to react in molecular proportions with some substance in globulin, either enzyme or inhibitor, during the activation of proteolytic enzyme. A small amount of fibrinolysin activated only a small proportion of the enzyme precursor. The following experiments were designed to test whether in the globulin preparation separated by isoelectric precipitation inhibitory substances were present which interfered with the complete activation of all available precursor.

Twenty cc. of pooled serum[1] was heated to 56°C. for 30 minutes, diluted with 19 volumes of water and sufficient 1 per cent acetic acid to bring the pH to 5.2. The resultant precipitate was separated by centrifugation and dissolved in 40 cc. of buffer. Two cc. of globulin solution was placed into each of 4 tubes and varying amounts of streptococcal fibrinolysin (previously dialyzed against cold distilled water and diluted with equal parts of buffer) were added. Sufficient buffer was added to each to bring the volume to 14 cc. The globulin-fibrinolysin mixtures were then incubated at 37°C. The proteolytic activity of the globulin-fibrinolysin mixtures was determined at the onset, and after incubation at 37°C. for 1 and 2½ hours by incubating 2 cc. aliquots with 2 cc. of 0.3 per cent casein at 37°C. for 1 hour. At the same time, the experiment was repeated with the same serum unheated.

When the globulin precipitated from serum which had been heated to 56°C. for half an hour was mixed with small amounts of streptococcal fibrinolysin, the amount of activated enzyme appeared to increase over a period of several hours (Fig. 5, *A*). Furthermore, when sufficient precursor was present, the amount of enzyme activated was directly proportional to the concentration of streptococcal fibrinolysin added. On the other hand, when streptococcal fibrinolysin was added to unheated serum (Fig. 5, *B*) the activity induced by fibrinolysin was maximal at the onset of the experiment and decreased during the period of observation. The same results were obtained by mixing plasma globulin solution with chloroform for 16 hours and removing the chloroform before the addition of streptococcal fibrinolysin.

These experiments imply either that heat or chloroform may activate directly or indirectly plasma proteolytic enzyme or, more likely, that they destroy some substance in the globulin preparation which inhibits the activation of enzyme by streptococcal fibrinolysin. The significance of these observations will be discussed below.

III. The Kinetics of Digestion with Plasma Proteolytic Enzyme

1. The Influence of Substrate Concentration on the Digestion of Casein by Plasma Proteolytic Enzyme.—To determine the influence of varying the con-

[1] Serum was used instead of plasma since heating plasma to 56°C. results in precipitation of fibrinogen.

centration of substrate upon the amount of substrate digested, chloroform-activated enzyme was prepared in the usual manner.

In a typical experiment, 4 cc. of chloroform-activated enzyme, prepared as in other experiments, was added to 4 cc. of casein dissolved in 0.006 N sodium hydroxide. The casein varied in concentration from 0.1 to 0.4 per cent, so that the final concentration of casein in the enzyme-substrate mixture was 0.5 to 2.0 mg. per cc.

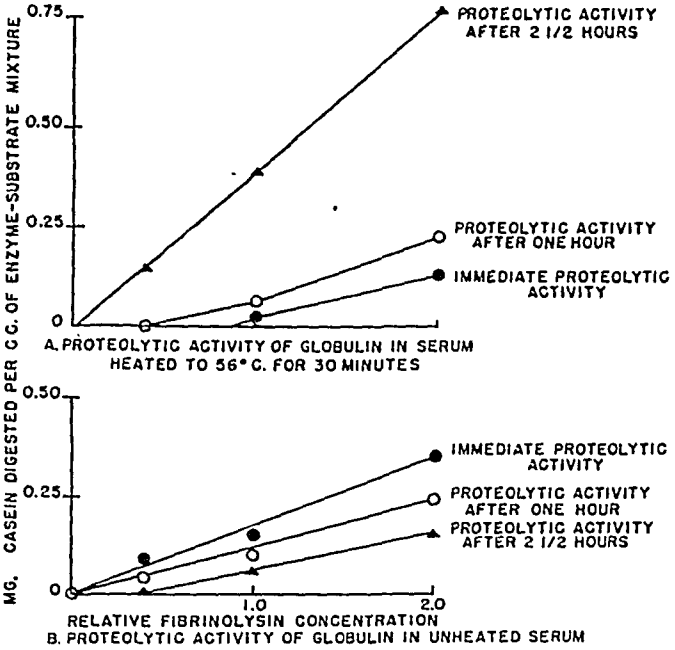

FIG. 5. The proteolytic activity induced by streptococcal fibrinolysin in globulin which had been heated to 56°C. Proteolysis was measured immediately after the addition of fibrinolysin, and again after incubation of the globulin-fibrinolysin mixture at 37°C. for 1, and 2½ hours. See text.

The amount of casein digested during a period of 4 hours is plotted in Fig. 6. During the first hour and a half, the amount of casein digested was independent of substrate concentration in those tubes with initial casein concentrations of 1.0 mg. per cc. or more. Beyond this time, however, the rate of digestion of casein was directly dependent upon the initial casein concentration.

These studies indicate that the quantitative determination of plasma proteolytic enzyme requires that a concentration of casein be used which would saturate all available enzyme during the period of digestion observed. However, in practice, a concentration of substrate higher than 1.5 mg. per cc. had to be avoided. If the concentration of casein was high relative to the concentration of globulin, when hydrochloric and sulfosalicylic acids were added to assay protein, flocculation and precipitation of protein resulted, instead of uni-

form turbidity. This rendered it impossible to quantify the protein present by the method used. Furthermore, concentrations of casein in the enzyme-substrate mixture higher than 1.5 mg. per cc. apparently inhibited digestion during the first 15 or 20 minutes of incubation. In repeated experiments, when concentrations of casein in the enzyme-substrate mixture of 1.5 mg. per cc. were used, the amount of casein digested was linear until approximately 0.7 mg. per cc. was digested, after which the amount of casein digested per unit time decreased.

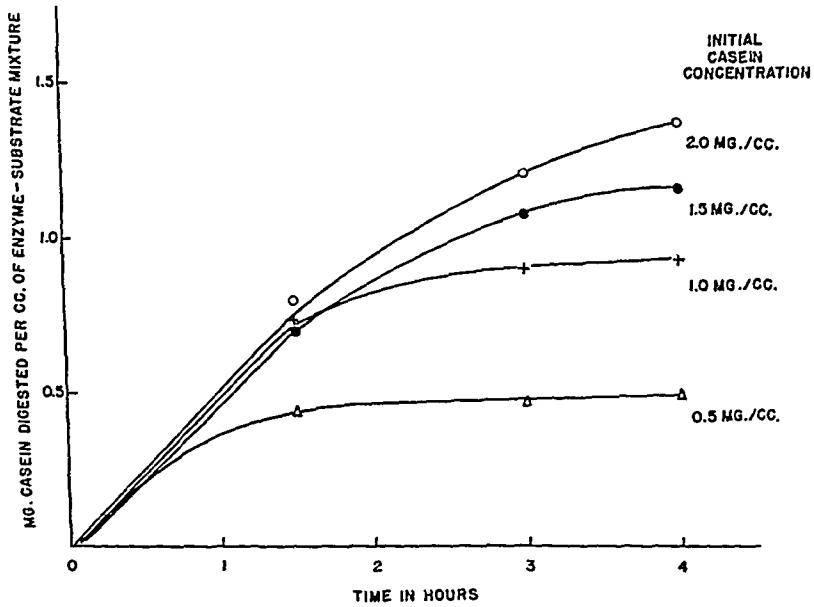

FIG. 6. The influence of the concentration of substrate (casein) on its rate of digestion by chloroform-activated plasma proteolytic enzyme. The initial concentration of casein is expressed in terms of milligrams per cubic centimeter of enzyme-substrate mixture.

2. The Relationship between Concentration of Enzyme and the Digestion of Casein during the 1st Hour of Incubation of Enzyme and Substrate.—When the initial concentration of casein in the enzyme-substrate mixture was 1.5 mg. per cc., digestion with either fibrinolysin- or chloroform-activated enzymes was proportional to the concentration of enzyme present, up to the point where 0.7 mg. per cc. of casein had been digested. Beyond this point, however, the relative rate of casein digested per cc. decreased.

Fig. 7 is a composite of many experiments in which the amount of casein digested by various dilutions of fibrinolysin-activated and chloroform-activated enzyme was plotted against arbitrary units. The amount of enzyme necessary to digest 0.50 mg. of casein per cubic centimeter of enzyme-substrate mixture

in 1 hour was arbitrarily called 50 digestion units. A sample of chloroform-activated enzyme was diluted to varying degrees, and the amount of casein per cubic centimeter of enzyme-substrate mixture digested in 1 hour was determined. The data from one such experiment were then plotted, using the arbitrary point of 50 units = 0.50 mg. as one point on the curve. Using this rough plot, the data from other experiments were fitted to the curve, selecting the best fit to the arbitrary curve. In this manner, it was possible to plot the data

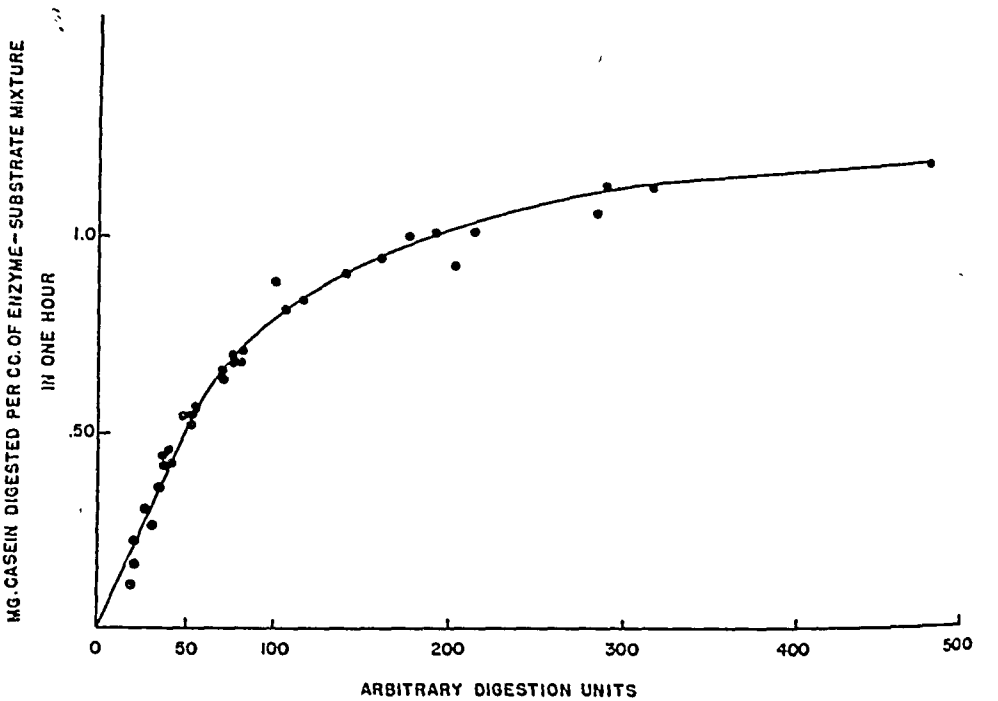

FIG. 7. The influence of the concentration of plasma proteolytic enzyme on the digestion of casein. The data of several experiments have been plotted on a single curve, with 50 digestion units arbitrarily designated as the amount of plasma proteolytic enzyme which will digest 0.50 mg. of casein per cc. of enzyme-substrate mixture in 1 hour, when the initial concentration of casein was 1.5 mg. per cc. of enzyme-substrate mixture. See text.

of many experiments in such a way that arbitrary units would express the proteolytic power of any given specimen. Thus, if a given solution digested 1.00 mg. of casein per cc. of enzyme-substrated mixture in 1 hour, it would contain 195 digestion units of plasma proteolytic enzyme. If the enzyme were diluted with equal parts of buffer, it would contain 97 digestion units and should digest 0.77 mg. of casein per cc. per hour. Actually, these assumptions have been found to be correct in repeated experiments. In this way, by the use of an arbitrary scale, the difficulties imposed by not using a substrate concentration higher than 1.5 mg. per cc. were overcome.

3. The Activity of Plasma Proteolytic Enzyme after the 1st Hour of Incubation

with Substrate.—In studying the activation of plasma proteolytic enzyme by streptococcal fibrinolysin, use was made of the fact that fibrinolysin-activated enzyme prepared from plasma globulin deteriorated rapidly at 37°C. This deterioration appeared to occur early during the 2nd hour after the enzyme had been activated. In these experiments, described in section II 4, the deterioration seemed to take place before the addition of substrate. The following experiments demonstrate that fibrinolysin-activated enzyme also deteriorated in

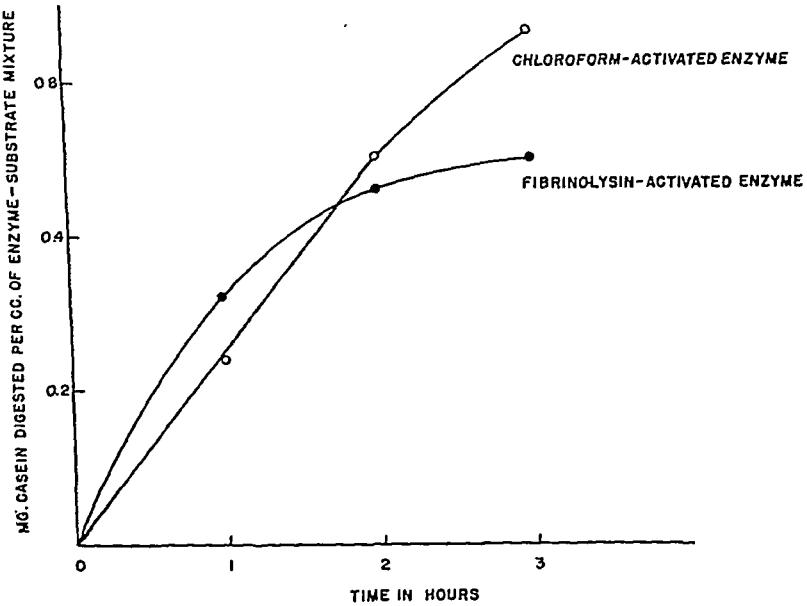

FIG. 8. The relative time of deterioration of chloroform-activated and fibrinolysin-activated plasma proteolytic enzyme. See text.

the presence of casein substrate. On the other hand, chloroform-activated enzyme did not deteriorate appreciably during a period of 3 hours.

The digestion of casein by samples of fibrinolysin-activated and chloroform-activated enzyme has been compared in Fig. 8. Chloroform-activated enzyme did not show appreciable deterioration during the 3 hour period. The rate of digestion decreased only after the amount of substrate fell below the concentration necessary to saturate all available enzyme; that is, when the concentration of casein decreased below approximately 0.8 mg. per cc. This indicates that there was no appreciable deterioration of chloroform-activated enzyme during the period of observation.

On the other hand, in this and many other experiments, the activity of fibrinolysin-activated enzyme decreased rapidly beyond the 1st hour of incu-

bation, regardless of how much casein was still available. This implies that the fibrinolysin-activated enzyme had deteriorated in one way or another. As pointed out before, this decrease in activity occurred even in the absence of substrate. The deterioration of fibrinolysin-activated enzyme occurred in all preparations studied, and became sufficient to affect the assay of proteolytic activity early during the 2nd hour of incubation at 37°C. Ordinarily, proteolytic activity was determined by measuring the amount of substrate digested in 1 hour. Therefore, it was necessary to assay fibrinolysin-activated enzyme immediately after preparation to minimize the effects of deterioration.

4. The Influence of Chloroform on the Deterioration of Plasma Proteolytic Enzyme Activated by Chloroform or by Streptococcal Fibrinolysin.—Chloroform-activated plasma proteolytic enzyme apparently did not deteriorate during several hours' incubation at 37°C. On the other hand, fibrinolysin-activated enzyme deteriorated rapidly. The following experiments were designed to

TABLE III

The Protection of Fibrinolysin-Activated Enzyme by Chloroform

Activator	Time of measurement of activity	Enzymatic activity
		*mg./cc.**
Fibrinolysin	Immediately after preparation	0.76
Fibrinolysin	After incubation at 25°C. for 20 hrs.	0.09
Chloroform	After incubation at 25°C. for 20 hrs.	0.09
Fibrinolysin and chloroform together	After incubation at 25°C. for 20 hrs.	0.60

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

determine whether chloroform removed or destroyed some substance which hastened the deterioration of plasma proteolytic enzyme.

In a typical experiment, the euglobulin precipitate of 14 cc. of plasma was prepared in two unequal portions. The precipitate of 2 cc. was dissolved in 1 cc. of buffer, and half a cc. of chloroform was added. This mixture was incubated for 20 hours at 25°C., and the chloroform-activated proteolytic activity was then determined in the usual manner. The euglobulin precipitate of the remaining 12 cc. of plasma was dissolved in 6 cc. of broth filtrate containing streptococcal fibrinolysin. It was demonstrated that this amount of fibrinolysin was not sufficient to activate all available precursor. The fibrinolysin-activated proteolytic activity was determined immediately on a 1 cc. aliquot. Another 1 cc. aliquot was allowed to stand at 25°C. for 20 hours, and its proteolytic activity then determined. To a third 1 cc. aliquot was added 0.5 cc. of chloroform and the mixture was allowed to stand at 25°C. for 20 hours, after which its proteolytic activity was determined. It was then possible to compare the proteolytic activity of the globulin immediately after the addition of fibrinolysin, 20 hours after the addition of fibrinolysin, 20 hours after the addition of fibrinolysin and chloroform together, and 20 hours after the addition of chloroform alone.

These data demonstrate that the addition of chloroform to fibrinolysin-activated enzyme immediately after it had been prepared, prevented in large meas-

ure the deterioration of plasma proteolytic enzyme over a period of 20 hours (Table III). It seemed of interest to determine whether chloroform would reactivate fibrinolysin-activated plasma proteolytic enzyme once it had deteriorated.

At the same time as the preceding experiment, two other 1 cc. aliquots of fibrinolysin-activated globulin were incubated at 37°C. for 4 hours. At the end of that time, one aliquot was tested for proteolytic activity. To the other aliquot was added 0.5 cc. of chloroform, the mixture was incubated for 16 hours at 25°C., and its proteolytic activity then determined. It was then possible to compare the proteolytic activity of the globulin immediately after the addition of fibrinolysin; after 4 hours at 37°C. following the addition of fibrinolysin; 16 hours after the addition of chloroform to enzyme which had been incubated for 4 hours at 37°C. after the addition of fibrinolysin; and 20 hours after the addition of chloroform alone.

As can be seen in Table IV, once fibrinolysin-activated enzyme had been allowed to deteriorate, the addition of chloroform did not reverse this deteri-

TABLE IV

Failure of Chloroform to Reverse the Deterioration of Fibrinolysin-Activated Enzyme

Activator	Time of measurement of activity	Enzymatic activity
		*mg./cc.**
Fibrinolysin	Immediately after preparation	0.76
Fibrinolysin	After incubation at 37°C. for 4 hrs.	0.06
Chloroform	After incubation at 25°C. for 20 hrs.	0.09
Fibrinolysin incubated at 37°C. for 4 hrs., then chloroform	After incubation at 25°C. for 16 hrs. following addition of chloroform	0.20

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 1 hour.

oration. The small increase in activity noted could be accounted for entirely by the activation of precursor not previously activated by fibrinolysin, since the precursor had not been saturated by the amount of fibrinolysin employed.

DISCUSSION

The experiments described define some of the conditions necessary for the optimal activation of plasma proteolytic enzyme by chloroform. Mixtures of chloroform and the euglobulin containing the precursor of plasma proteolytic enzyme were incubated for varying lengths of time and at several temperatures. The greatest proteolytic activity resulted when the plasma euglobulin was incubated with chloroform for 16 to 28 hours at 25°C. This is in contradistinction to the reported results of incubation of chloroform and whole plasma (3, 9), mixtures of which were not maximally active for at least several days. These studies suggest that some substances not in euglobulin may retard the activation of plasma proteolytic enzyme. Incubation of globulin with chloroform at 37°C. resulted in much less proteolytic activity than incubation at 25°C. The explanation of this phenomenon is not clear, although it is possible that

the enzyme may be destroyed by the prolonged incubation at 37°C., perhaps by autodigestion.

The evidence presented did not indicate the mode of activation of plasma proteolytic enzyme by chloroform. Delezenne and Pozerski (3) and later Christensen (9) suggested that chloroform apparently removes some substance which prevents the accumulation of enzyme. Then the enzyme may be activated autocatalytically in the manner in which trypsinogen is activated by trypsin (9). The data support this view but the evidence is not conclusive.

Streptococcal fibrinolysin on the other hand, activated plasma proteolytic enzyme with great rapidity and when maximal amounts of fibrinolysin were used, maximal activity was observed just as early as proteolysis could be measured. Christensen and MacLeod (7) studied the proteolysis of gelatin with a purified streptococcal fibrinolysin prepared from the H46A strain of beta hemolytic streptococcus. They concluded that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin was a catalytic process. The rate of activation was proportional to the concentration of fibrinolysin, but the maximal activity developed was independent of the concentration of fibrinolysin used.

Moreover, Christensen and MacLeod (7, 9) have proposed an hypothesis which explains the data thus far presented concerning the rôle of chloroform and of fibrinolysin in the activation of plasma proteolytic enzyme. Plasma proteolytic enzyme is capable of spontaneous activation. Chloroform removes some substance which destroys the plasma proteolytic enzyme as it is formed. Therefore, when globulin solutions are treated with chloroform, the spontaneously developing enzyme gradually accumulates, and the preparation acquires considerable proteolytic activity. Fibrinolysin, on the other hand, reacts catalytically directly with some substance in globulin to release plasma proteolytic enzyme.

Other data, reported above, however, are not adequately explained by this hypothesis. If the globulin preparation was not previously treated with heat or chloroform, the activation of plasma proteolytic enzyme with streptococcal fibrinolysin behaved as if a stoichiometric reaction occurred. Thus, the proteolytic activity evoked by fibrinolysin acting on plasma globulin preparations not so treated was an hyperbolic function of the concentration of fibrinolysin. This is compatible with the law of mass action. Furthermore, if the concentration of both fibrinolysin and globulin was varied, the proteolytic activity that resulted was proportional to the product of the concentration of fibrinolysin and of globulin. That is, within limits,

$$\frac{[\text{Substance in globulin}] \times [\text{fibrinolysin}]}{[\text{Proteolytic activity}]} = \text{Constant}$$

With the globulin preparation used, the enzymatic activity of fibrinolysin-globulin mixtures began to deteriorate after incubation for about 1 hour at

37°C. This was equally true if small or large amounts of fibrinolysin were used. When preparations of fibrinolysin-activated enzyme were allowed to deteriorate, the addition of fresh fibrinolysin evoked fresh enzymatic activity in those preparations which had low concentrations of fibrinolysin originally, and none in those which had high concentrations of fibrinolysin originally. Therefore, these experiments indicate that fibrinolysin reacted in molecular proportions with some substance in globulin, probably the precursor of plasma proteolytic enzyme itself.

All the data above can be explained by a new hypothesis. The globulin preparation used contained small amounts of inhibitor of activated plasma proteolytic enzyme. Streptococcal fibrinolysin reacts stoichiometrically with the precursor of plasma proteolytic enzyme. In the absence of these inhibitory substances, the enzyme so activated is responsible for further autocatalytic activation of the remaining enzyme precursor. In the presence of inhibitor of the activated enzyme, the autocatalysis is blocked, and the molecular reaction is then apparent.

Certain data described above support this hypothesis. When the plasma globulin was treated with chloroform or heat, streptococcal fibrinolysin now appeared to activate plasma proteolytic enzyme catalytically. However, the globulin thus treated simultaneously lost its ability to inhibit plasma proteolytic enzyme (2). Furthermore, fibrinolysin-activated enzyme prepared from globulin treated with heat or chloroform did not deteriorate with the rapidity of unheated globulin preparations. These experiments suggest that the inhibitory substance which prevents the catalytic activation of plasma proteolytic enzyme may be identical with the inhibitor of activated plasma proteolytic enzyme.

The kinetics of digestion with plasma proteolytic enzyme have not been defined previously. The data presented here demonstrate that the relationship between concentration of enzyme and the amount of substrate digested is similar to that of other hydrolytic enzymes. When the concentration of substrate is sufficiently high to saturate all available enzyme, the rate of digestion by hydrolytic enzymes is directly proportional to the concentration of the enzyme, and to the duration of digestion (10). This is particularly true early in the course of digestion, before the accumulation of inhibitory end products, or a decrease in the amount of substrate available, or the inactivation of the enzyme. The experiments herein described demonstrate that plasma proteolytic enzyme does indeed digest casein in direct proportion to the concentration of enzymes as long as the enzyme is saturated with substrate.

SUMMARY

1. Some conditions for the optimal activation of plasma proteolytic enzyme by chloroform have been described. The activation proceeds slowly. The action of chloroform is probably to remove some substance which inhibits or

inactivates the plasma proteolytic enzyme preparation, rather than a direct activation of the enzyme.

2. Plasma proteolytic enzyme is activated by filtrates of cultures of beta hemolytic streptococci. When streptococcal fibrinolysin is present in maximally effective amounts, the activation is almost instantaneous. When the globulin is prepared from heated serum or the globulin is treated with chloroform, the activation of enzyme by streptococcal fibrinolysin appears to be catalytic. If the globulin is not so treated, the reaction appears to involve a stoichiometric process.

3. The plasma proteolytic enzyme activated by chloroform or by streptococcal fibrinolysin digests casein in direct proportion to the concentration of enzyme and to the time of digestion, during the early period of incubation.

4. Fibrinolysin-activated enzyme deteriorates rapidly relative to chloroform-activated enzyme. This may be due to the removal by chloroform of some substance which inactivates plasma proteolytic enzyme.

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THE PROTEINS IN UNHEATED CULTURE FILTRATES OF HUMAN TUBERCLE BACILLI

I. Fractionation and Determination of Physical-Chemical Properties*‡

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INTRODUCTION

Different preparations of P.P.D. (purified protein derivative) (1) and of O.T. (old tuberculin) (2) tuberculins are known to differ in their skin activity. Since the latter property is associated wholly with the protein component of tuberculins (3), a partial explanation of this phenomenon is that the protein contents of lots of O.T. may be significantly different, but this can hardly account for the variation found in preparations of P.P.D. (3). The presence of two or more proteins with unequal skin activities is one possible explanation, as they might be found in varying amounts in different preparations; or, as has been suggested, degradation of the protein during preparation might account for the observed facts (3).

In an attempt to get a complete picture of the proteins in tuberculin, we have fractionated with ammonium sulfate unheated culture filtrates of both a virulent strain of human tubercle bacillus and a strain of low virulence, and studied the protein fractions both serologically and by physical-chemical methods.

That two or more proteins exist in tubercle bacilli or in tuberculins has been indicated by other workers (4), but complete separation and proof of antigenic individuality have not been obtained. By means of electrophoresis Seibert and Nelson (5) have demonstrated in unheated culture filtrates of tubercle bacilli protein-containing materials of at least two different mobilities. Two of the fractions separated thus had some immunological specificity but cross-reactions were obtained. Furthermore, one fraction was found to be much less antigenic than the other, and it failed to sensitize the skin of rabbits. Thus it was not clear that the one was not a degraded form of the other.

* The physical-chemical work is more fully described in the thesis of one of the authors submitted to the Faculty of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree in June, 1944.

This phase of the investigation was carried out in the laboratories of Dr. J. W. Williams, and we wish to express our appreciation of his help.

‡ Supported in part by grants from the Wisconsin Alumni Research Foundation and from the National Tuberculosis Association Medical Research Committee.

We have found, principally from the results of sedimentation velocity experiments, that there are two distinct proteins with differing sedimentation constants in tuberculin, one of which is the same as that obtained previously by Seibert *et al.* (6) by ammonium sulfate fractionation. Our data indicate a slightly higher molecular weight for this protein than the 32,000 determined by Seibert *et al.* The serological work has confirmed the individuality of the two proteins of different sedimentation constant and has, in addition, established the presence of a third, serologically distinguishable factor not detected in the physical-chemical experiments. Additional tests, including active skin sensitization in guinea pigs and passive skin sensitization of normal guinea pigs with serum from the animals actively sensitized, have further demonstrated the biological specificity of the two proteins and shown that both have similar degrees of antigenicity.

The present paper describes the fractionation of the unheated culture filtrates and the physical and chemical investigation of the products. The serological studies and a description of the skin activities of the fractions will be presented in the second paper of this series (7).

EXPERIMENTAL

The cultures were grown on Henley's synthetic medium (8), the virulent strain (DT)[1] for 21 weeks and the slightly virulent one (TB–1)[2] for 9 weeks. Thereafter the cultures of the two strains were treated in exactly the same way. Cellular material was removed by filtration through Mandler filters. The filtrates were immediately concentrated and washed by ultrafiltration (1) with phosphate buffer (0.005 M, pH 7.4 to 7.5) at 5°C. Throughout the process merthiolate was maintained at a concentration of 1/10,000 to prevent bacteria from growing, and both the ultrafiltration and the subsequent fractionation were carried out at 0–5°C. to avoid denaturing the protein and also to prevent contamination. To ascertain whether any significant constituents were being lost by ultrafiltration, parts of the ultrafiltrates were in turn concentrated and washed in the alundum cups impregnated with heavier cellulose acetate films. All preparations were lyophilized immediately after they had been washed by ultrafiltration.

The fractionation scheme is given in Fig. 1 and the amount obtained and chemical composition of each fraction are in Tables I and II. Corresponding fractions of the two strains have the same symbol; "av" is affixed to designate those from the slightly virulent tubercle bacillus, and "v" to indicate those from the virulent strain.

All precipitations were carried out according to the following scheme. A solution containing approximately 1 per cent protein was dialyzed in several changes of the precipitating solution, usually an ammonium sulfate solution 0.25, 0.5, or 0.75 saturated at 5°C., either at the normal pH or neutralized with sodium hydroxide to pH 7.2 as indicated in Fig. 1. After about 24 hours the precipitate in the dialysis bag was centrifuged. The supernatant liquid

[1] The culture DT was obtained through the courtesy of Dr. Florence B. Seibert. Its virulence was checked in guinea pigs just before the culture filtrates were prepared from it.

[2] TB–1 is the number of the stock culture of the Department of Agricultural Bacteriology of The University of Wisconsin.

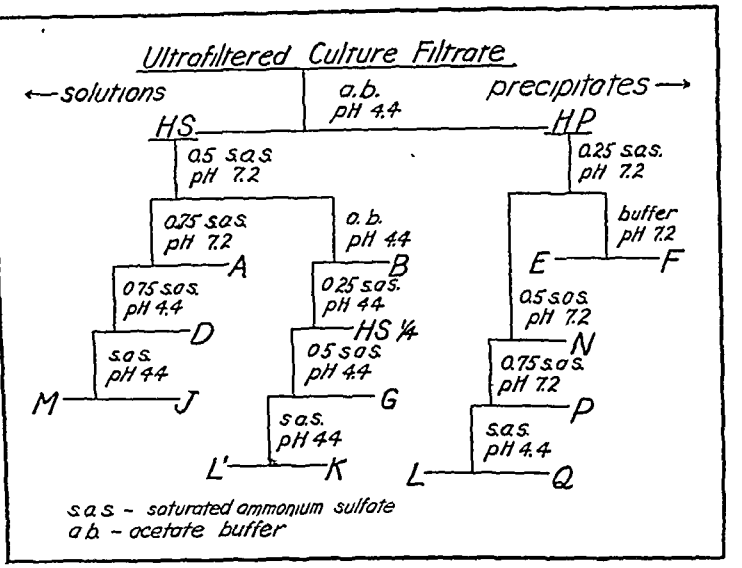

FIG. 1. Fractionation scheme for unheated culture filtrates.

TABLE I

Composition and Yield of Tuberculin Fractions from Strain DT

Fraction	Protein	Polysaccharide	Nucleic acid*	Total yield of protein‡
	per cent§	*per cent*	*per cent*	*mg.*
HS	32	68		3,380
HP	69	11	20	775
B	86	14	0	16
HS¼	94	6	0	119
G	87	12	0.5	115
K	69	28	3	20
L'	10	89	1	3
A	71	29		332
A reprecipitated	91	8	1	99
D	72	27	1	202
J	27	55	18	106
M	5.5	94	0.5	95
E	87	5	8	112
N	94	2	4	154
P	78	8	14	180
Q	23	25	52	22
L	17	1	82	12

* Nucleic acid values of less than 15 per cent are not accurate due to interference of protein and polysaccharide.

‡ The original volume of the culture medium was 12 liters.

§ Per cent of the sum of the three substance for which analyses were made.

was poured off and dialyzed with the next precipitant. The precipitate was washed at least once with the precipitating solution and recentrifuged. It was then dissolved in 0.02 M phosphate buffer of pH 7.0 to 7.2, and dialyzed in frequent changes of this buffer until all ammonium sulfate was removed. Determinations of polysaccharide, nitrogen, and nucleic acid were made on this solution.

Following Seibert (9), the nucleic acid contents of the fractions were estimated by the diphenylamine reaction of Dische, and the polysaccharide contents by the carbazole reaction. We are indebted to Dr. Florence B. Seibert for a sample of Hammarsten's pure sodium thymonucleate and also for a sample of purified tuberculin polysaccharide, which were used as

TABLE II

Composition and Yield of Tuberculin Fractions from Strain TB-I_{av}

Fraction	Protein	Polysaccharide	Nucleic acid	Total yield of protein*
	per cent	*per cent*	*per cent*	*mg.*
HS	29	71		1,034
HP	92	5	3	232
B	86	14	0	53
HS¼	94	6	0	65
G	84	16	0	43
K	69	30	1	6
L'‡	85	15		1.5
A	75	24	0.5	232
A reprecipitated	88	12	1	57
D	73	27	0.5	43
J	41	59	0.4	47
M§	7	93	0	54
E	83	12	4	9
N	93	6	0.7	15
P	85	11	4	26
Q	85	9	6	11
L‡	54	46		1.6

* The original volume of the culture medium was 10 liters.

‡ Total amount too small to determine nucleic acid.

§ Dialysis bag broke and some material was lost. Total amount, therefore, in error but percentages correct.

standards in the determinations. Two or more concentrations of each standard were run concurrently with each set of determinations to serve as a check on reagents and methods. Nevertheless, for fractions containing appreciable amounts of both nucleic acid and polysaccharide such colorimetric determinations are not accurate. The nucleic acid gives a characteristic color with carbazole (10) which is quite different from the brownish pink given by the tuberculin polysaccharide. A polysaccharide analysis using the carbazole reagent on pure nucleic acid showed that at the wave length of light used in the analysis, the nucleic acid absorbed twice as strongly as did the polysaccharide. The polysaccharide analyses given in Tables I and II are, therefore, corrected for nucleic acid by subtracting twice the nucleic acid value from the polysaccharide value. The nucleic acid values themselves are questionable, however, on samples containing small amounts of nucleic acid and large amounts of either protein or polysaccharide. It was found that if the nucleic acid nitrogen were less than 15

per cent of the total nitrogen, the color developed by the reagent was greenish blue instead of violet, and the relationship between the amount of sample and the logarithm of the fraction of light absorbed was no longer linear. The ratio of the amount of light absorbed using a 660 mμ filter to that with a 620 mμ filter for the fractions low in nucleic acid was compared with the same ratio for pure nucleic acid, and incorrect ratios were obtained for all containing less than 15 per cent nucleic acid. Protein contents were estimated from nitrogen determinations (11) by using the value 16.3 for the percentage of nitrogen in tuberculin proteins (6), and by correcting for nucleic acid nitrogen.

The fractionation of the concentrated and washed culture filtrates was greatly complicated by the presence of polysaccharide, often amounting to 70 per cent of the total material in solution, and of nucleic acid in the "v" preparations. The nucleic acid was almost entirely separated by adjusting the solution to pH 4.4, whereupon a heterogeneous protein-nucleate and any denatured protein precipitated. The precipitate, HP in the fractionating scheme, was further fractionated, as it contained considerable protein. Fractions E, N, and P were obtained by successive precipitations with 0.25, 0.5, and 0.75 saturated (at 5°C.) ammonium sulfate brought to pH 7.2 with NaOH, at which pH more protein than nucleic acid is precipitated by ammonium sulfate (9). A final precipitation at pH 4.4 with saturated ammonium sulfate brought down the remaining protein leaving only a few milligrams in solution. Part of the 0.25 HP fraction (F) would no longer dissolve in phosphate buffer after removal of the ammonium sulfate. Although it dissolved in a borate buffer of pH 9, it precipitated after a few days and was discarded.

Most of the protein remained in the solution (HS) after the initial precipitation at pH 4.4, together with nearly all the polysaccharide. Precipitation at pH 7.2 with 0.5 saturated ammonium sulfate brought down a protein fraction containing 35 per cent polysaccharide. To lower the polysaccharide concentration, the further precipitations were carried out at pH 4.4, near the isoelectric point of the tuberculin proteins. The assumption was that at this pH the solubility of the protein would be a minimum, while that of a neutral polysaccharide would be unchanged. The fraction was dissolved in phosphate buffer, adjusted to pH 4.4 in acetate buffer by dialysis, and precipitated with 0.25 saturated ammonium sulfate. A small amount which precipitated in the acetate buffer was collected by centrifugation as fraction B. The fractions from both strains were found to contain about 10 to 15 per cent polysaccharide. A second precipitation of the protein at a concentration of about 0.3 per cent yielded a fraction in each case containing only 6 per cent polysaccharide at a sacrifice of about one-third of the protein. Further decrease in the polysaccharide content was impractical because of the large loss of protein.

The supernatant solutions from these precipitations and, separately, that from the 0.5 saturated ammonium sulfate precipitation, were further fractionated as indicated in Fig. 1. The final solution remaining contained fraction M, almost pure polysaccharide. The A fractions, containing a relatively large amount of material in both preparations, were reprecipitated from dilute (about 0.3 per cent) solution and fractions containing 90 per cent protein were obtained.

All the fractions in which there was sufficient material were sedimented in the Svedberg oil-driven ultracentrifuge at 60,000 R.P.M. in a 0.02 M phosphate buffer, 0.2 M in sodium chloride, at a pH of 7.0. Diagrams of the scale line displacement, which is proportional to the concentration gradient, *versus* distance in the cell are given in Figs. 2 *a*, *b*, and *c* for some of the fractions together with the sedimentation constants calculated therefrom. The constants for the pure fractions are s°_{20} values, having been corrected to 20°C. and to a process taking place in pure water. This last correction, which amounts to 3 or 4 per cent, is meaningless for mixtures as it involves the partial specific volume of the solute, and so only the density correction has been made for the impure fractions and their constants are thus given as s_{20} values. Since little information is obtained from one experimental curve, several for each

individual experiment are given. These show the changes in concentration gradients in the cell with time. The two proteins found were detected by a combination of this information with that obtained from the chemical analyses, as shown in the following description of the fractions.

Fraction HS¼.—Ten sedimentation experiments were made on five different preparations of fraction HS¼, the purest protein fraction, at concentrations of 0.25 to 1.0 per cent. Curves from representative experiments are shown in Fig. 2 *a*. No change of sedimentation constant with concentration was observed. All the peaks were symmetrical and this fact, together with the low polysaccharide content, indicates that the fraction is probably a fairly homogeneous protein. The mean of the sedimentation constants for all experiments was 3.4 S with a precision of about 5 per cent.

Four diffusion experiments were made with two preparations of $HS¼_V$ at concentrations from 0.25 to 0.5 per cent, using a Lamm-Polson micro diffusion cell, and the Lamm scale method for observing the rate of diffusion (12). The low concentrations used made the experimental error in individual values of the diffusion constant rather large. The mean value of D^o_{20}, 7.3×10^{-7} cm.^{2} per second, has a precision of about 10 per cent. The experimental curves showed little or no deviation from the normal curve: thus no gross inhomogeneity with respect to diffusion constant existed in the preparation. This test for homogeneity is not sensitive to variation in diffusion constant of the order of 100 per cent (13).

The apparent partial specific volume of HS¼ was calculated from the results of very careful density measurements.[3] Three values, at concentrations of 0.276, 0.360, and 0.502 per cent, were obtained. The mean value, 0.738, had a probable error of less than 1 per cent.

By use of the values of partial specific volume ($\bar{V}$), diffusion constant (D^o_{20}), and sedimentation constant (s^o_{20}) the frictional ratio, f/f_0, was calculated:

$$f/f_0 = \frac{10^{-3}(1 - \bar{V}\rho)^{1/3}}{(D^2 s \bar{V})^{1/3}}$$

This ratio compares the frictional resistance of the molecules to sedimentation and diffusion with that of spherical molecules with the same molecular weight. From it the ratio of the minor semi-axis, b, to the major semi-axis, a, of the molecule was calculated, using Perrin's expression

$$f/f_0 = \frac{\left(1 - \frac{b^2}{a^2}\right)^{1/2}}{\left(\frac{b}{a}\right)^{2/3} \log \frac{1 + \left(1 - \frac{b^2}{a^2}\right)^{1/2}}{\frac{b}{a}}}$$

[3] Details of the determination of partial specific volume have been published (18, page 17). The method used was similar to that described by Tennent and Vilbrandt (16).

This applies exactly to a prolate ellipsoid of revolution, which is a sufficiently accurate approximation to the shape of a protein molecule in solution.

The molecular weight of HS¼, from the equation

$$M = \frac{RTs}{D(1 - \bar{V}\rho)}$$

together with the values of f/f_0 and a/b, is given in Table III. HS¼ appears to be a globular, hence probably undenatured protein. It is also evident, both from the values of the constants and from the method of preparation, that the HS¼ protein corresponds to a protein reported by Seibert *et al.* in 1938 (6). The values of s^o_{20} and D^o_{20} given for her preparation TPA-30b are 3.3 S and 8.2×10^{-7} respectively. These together with a value for the partial specific volume of 0.7 give a molecular weight of 32,000, compared with 44,000

TABLE III

Molecular Weights of Tuberculin Components

Material	s^o_{20} (S)	$D^o_{20} \times 10^7$ cm.²/sec.	$\bar{V}$	Molecular weight	f/f_0	a/b
HS¼$_V$	3.4	7.3	0.738	44,000	1.25	5
HS¼$_{aV}$*	3.4		0.738	44,000		
Polysaccharide M$_V$	1.7	7.6	0.619‡	18,000	1.71	13
Polysaccharide M$_{aV}$	2.1	7.0	0.619‡	23,000	1.71	13

* There was not enough material available to carry out diffusion experiments on HS¼$_{aV}$.
‡ Value of Seibert *et al.* (6).

for HS¼. The difference between the two molecular weights lies in the difference in the partial specific volume. The probable error in our value of $\bar{V}$ is less than 1 per cent and the probable error in the molecular weight of HS¼ is only about 10 per cent.

Fraction B (Fig. 2 *a*) appeared to be HS¼ which had undergone a change in solubility, precipitating at pH 4.4. Its sedimentation constant was 3.2 S, but the curves were not as regular as those for HS¼ and indicated a spread of molecular sizes or shapes. The change in solubility occurred rather rapidly in solutions of HS¼ at room temperature, much more slowly at 5°C. Presumably it came about very rapidly when the tuberculin was heated; when a culture filtrate heated for 2 hours was fractionated according to the scheme in Fig. 1, no precipitate appeared when the solution was dialyzed in 0.25 saturated ammonium sulfate.

It should be mentioned in this connection that measurements of the diffusion constant of HS¼ were carried out at 25°C. In the future it would be better to conduct diffusion experi-

ments at a lower temperature, near 0°C., to minimize the danger of denaturing the protein during an experiment. However, the time required for a diffusion experiment was much less than that for the first appearance of a precipitate at room temperature, so that the change in the measured diffusion constant due to denaturation was less than the experimental error inherent in the method.

HS Fractions Containing the 2 S Protein.—When the sedimentation curves for the other fractions were examined, it became evident that a clear cut separation of HS¼ from the rest of the protein had not been achieved in all these fractions. In most of the predominantly protein fractions derived from the acetate-soluble portion from the initial precipitation (A, D, and G, Fig. 2 *b*) the peaks were unsymmetrical and gave evidence of one component with a sedimentation constant of about 3.4 S, obviously HS¼, and another with a constant of about 2 S. In no case were the peaks sufficiently resolved so that accurate constants for both components could be calculated from one set of curves, but in several cases, fractions A_v and A_{av} (Fig. 2 *b*), for example, the faster protein predominated in the "v" preparation, determining the value of the constant, and the slower in the "av". Also in two different preparations of G_v, the faster component was in excess in one sample and the slower in the other, making it possible to calculate constants of 3.4 and 2.2 S respectively (Fig. 2 *b*). The latter value was also found in G_{av}. This sample gave remarkably symmetrical peaks on sedimentation, although it contained 16 per cent polysaccharide. This, together with the fact that all fractions in which the slower protein predominated, no matter what the polysaccharide content, showed sedimentation constants in the neighborhood of 2 S, leads us to believe that the sedimentation constant of the slower protein is very near that of the polysaccharide.

Fraction K_v (Fig. 2 *b*) and fractions J_v and J_{av} are important in that they are the only 2 S fractions which the serological work showed to contain no HS¼. Sedimentation experiments on J_v or J_{av} did not seem to be warranted because of the high polysaccharide contents of these fractions. No physical-chemical or serological work was done with the K_{av} preparation as there was so little material available. While K_v contained almost 30 per cent polysaccharide in addition to a small amount of nucleic acid, it gives the best indication of the sedimentation constant of the 2 S protein. The fact that the curves are slightly asymmetric, with spreading on the faster side, may indicate that the polysaccharide sediments slightly faster than the protein, but this spread may also be due to the presence of nucleic acid. The constant of 1.9 is due principally to the 2 S protein, and is probably not far from the value that would be found for pure 2 S protein containing no polysaccharide.

Polysaccharide.—Fraction M, the polysaccharide, was the only fraction, other than HS¼, that could be called a pure substance. It contained some nitrogen, amounting to about 6 per cent protein, if one assumes all the nitrogen to be in a protein impurity, but its sedimentation curves were as regular as

those of HS¼, yielding a value for s°_{20} of 1.7 S for M_v and 2.1 S for M_{av} (Fig. 2 *c*). It is not known at present whether the difference in the two values is real or merely indicates a need for more experimental data. Since the polysaccharide appeared to be homogeneous in its sedimentation behavior, and the amount of protein impurity was small, diffusion experiments were carried out, which, together with the partial specific volume of 0.619 determined by Seibert *et al.* (6), gave sufficient data to calculate the molecular weight and shape. These data are given in Table III. Because of the relatively low diffusion constant the molecular weight of the polysaccharide is greater than most values reported previously for tuberculin polysaccharides (14, 15).

HP Fractions.—A close similarity had been found in the sedimentation curves of corresponding fractions of the "v" and the "av" preparations obtained from the acetate-soluble fraction, HS, of the initial precipitation. The chief difference lay in the larger proportion of HS¼ found in the various fractions from HS_v. A correspondence was not found, however, among the fractions derived from the two precipitates, HP_v and HP_{av}. Fraction E_v exhibited fairly symmetrical curves with a constant of about 4.6 S, while E_{av} had scarcely any peak (Fig. 2 *c*). The curves for the latter spread badly, and the constant calculated from the maxima of the successive broad peaks was between 2 and 3 S. Fractions N_v and P_v, predominantly protein, but containing 5 and 15 per cent nucleic acid respectively, both had constants of 5.1 S, and produced curves which approached those typical of nucleic acids (16), having a long slope on the left-hand side (towards the top of the cell) and a sharp drop on the right-hand side (Fig. 2 *c*). Fraction Q_v, 50 per cent nucleic acid, showed this behavior also, but here it was much more pronounced (Fig. 2 *c*). The material responsible for the constant of 5 S could not be detected in E_{av}, N_{av}, or P_{av}. Since the serological work showed the HP fractions to contain only those proteins found in the HS fractions, none of which had constants larger than 3.5 S, the presence of this material seemed to be correlated with the relatively larger amount of nucleic acid in HP_v, 20 per cent, contrasted with the 3 per cent present in HP_{av}. This in turn was possibly connected with the length of autolysis of the two cultures, 21 weeks for the "v" and 9 weeks for the "av". It should be mentioned that the material responsible for the constant of 5 S was not an artifact arising from the method of fractionation, as peaks with this constant were observed in H_v, the concentrated culture filtrate itself, and in HP_v, the fraction that precipitated from acetic acid buffer at pH 4.4 (Fig. 2 *a*).

In the sedimentation of fractions E_v and N_{av} (Fig. 2 *c*) a second, very fast peak could be seen. In E_v, at a concentration of 1.2 per cent, its constant was about 15 S, while at a concentration of 0.3 per cent its value was about 20 S. In N_{av} the peak was extremely well defined, and had a constant of 19.3 S. Whether the material responsible for these peaks was native to the tuberculin or was formed during the course of fractionation cannot be said.

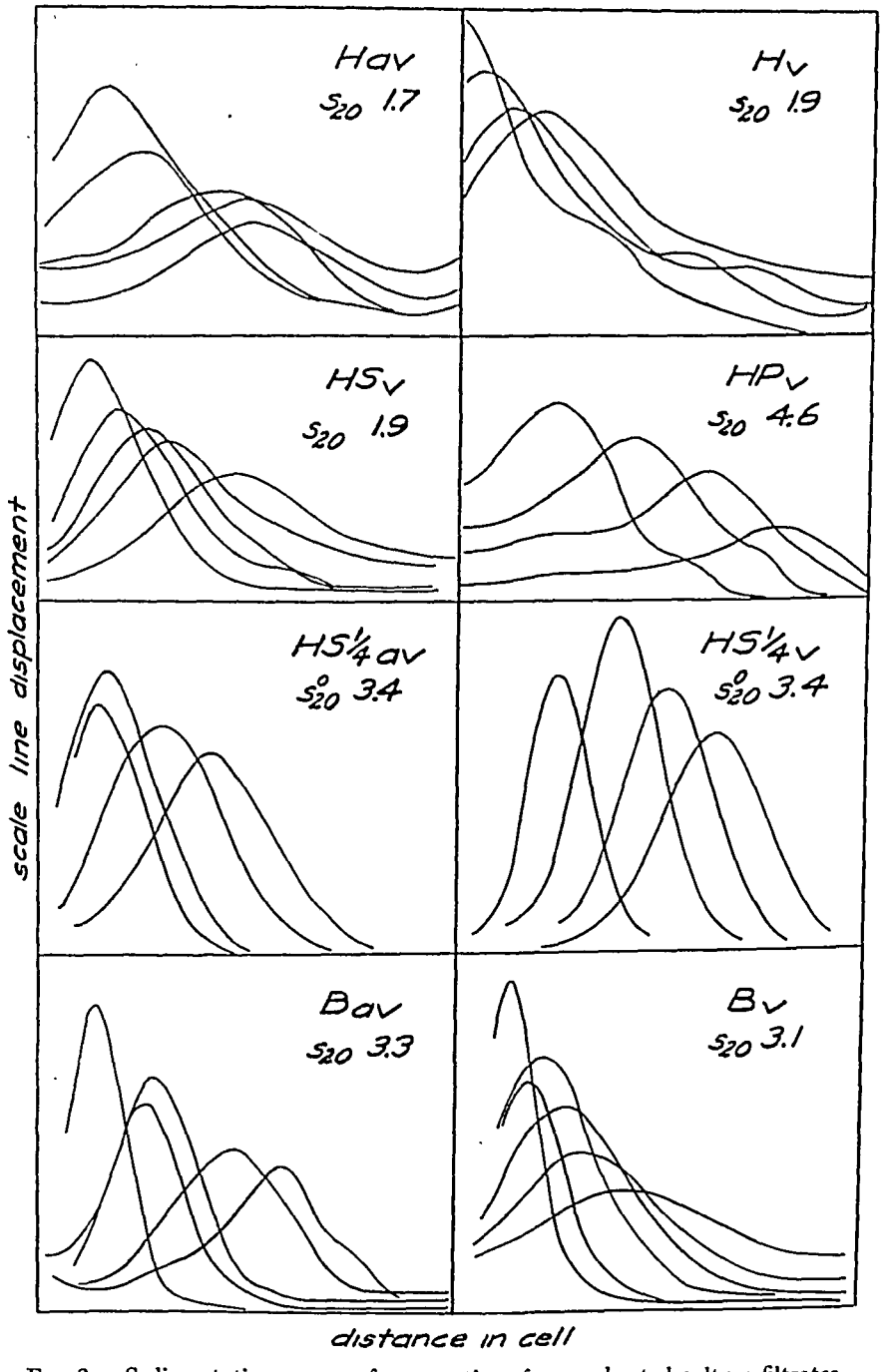

FIG. 2 *a*. Sedimentation curves of preparations from unheated culture filtrates.

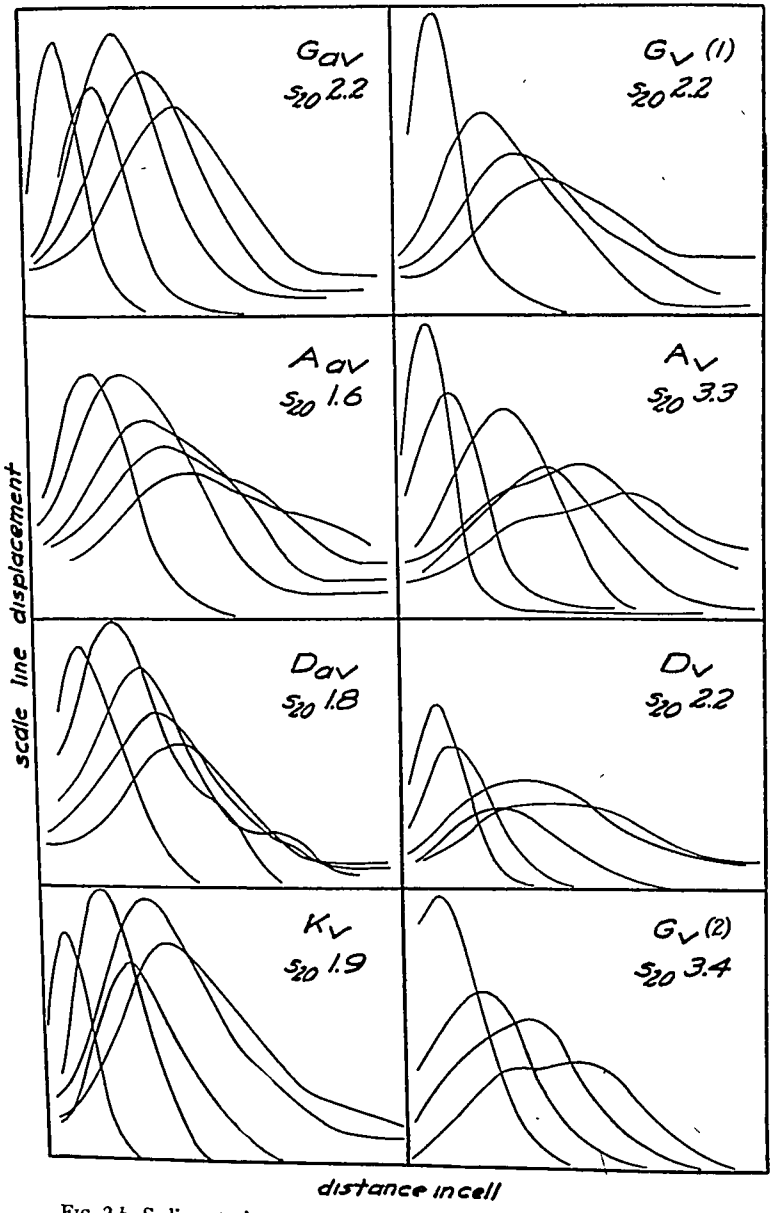

FIG. 2 *b*. Sedimentation curves of fractions from unheated culture filtrates.

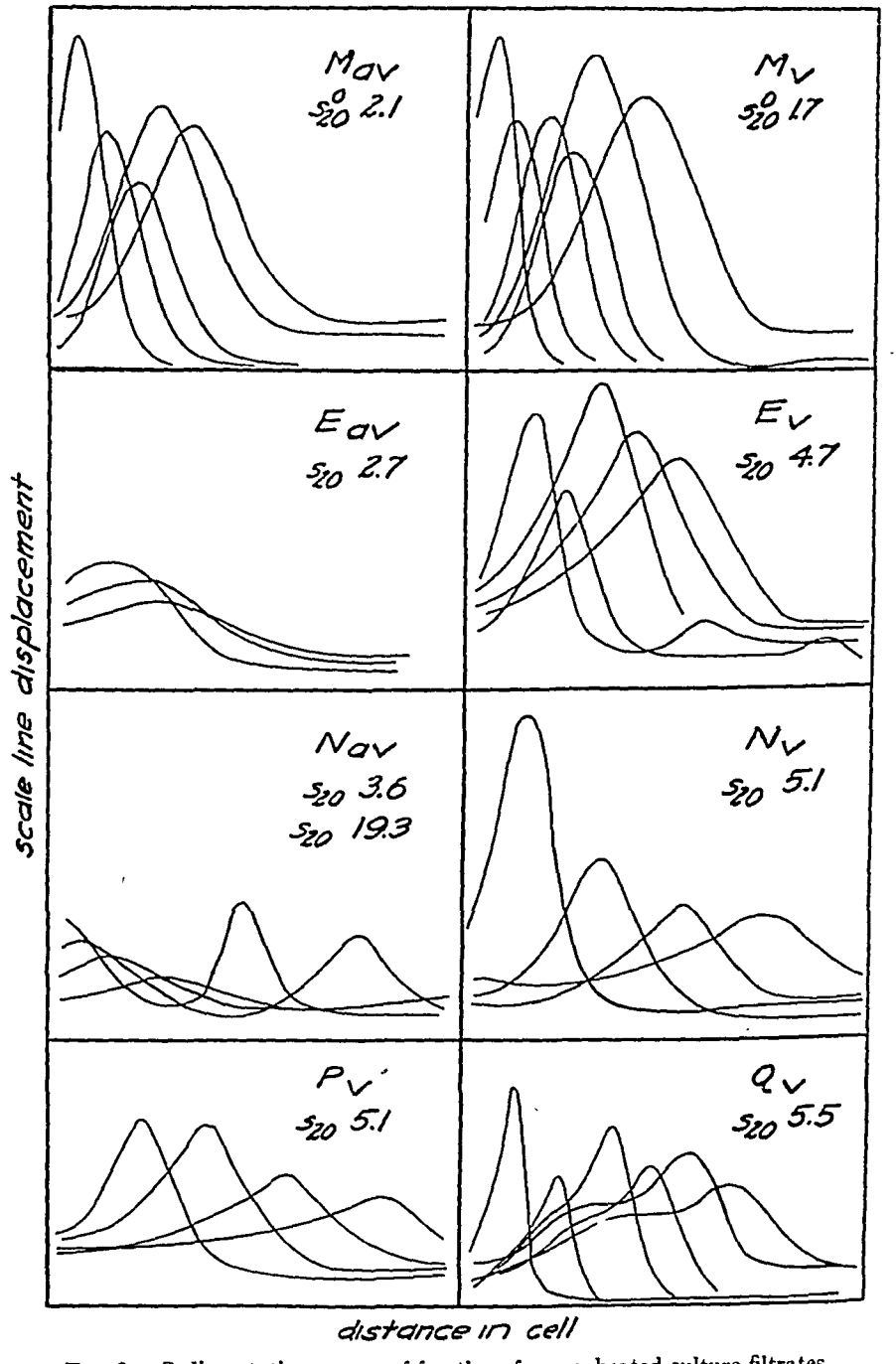

FIG. 2 *c*. Sedimentation curves of fractions from unheated culture filtrates.

DISCUSSION

We have obtained in the fractionation and sedimentation of unheated tubercle bacillus culture filtrates reliable evidence of only two proteins. The first, HS¼, can be described by its physical constants, since it was obtained in a relatively pure form. The second protein, present in almost all the other fractions from HS, was obtained free from HS¼ but in all cases contaminated with polysaccharide. It is probable that its sedimentation constant is close to 2 S. The small amount of material found in fractions E_v and N_{av} with a sedimentation constant near 20 S is suspected of being an artifact. The substance giving the peaks with a constant near 5 S in the fractions from HP_v appears from the immunological work to be a mixture of the proteins found in the HS fractions, so that it is probably not a distinct molecular species.

The nature of the peak with sedimentation constant varying from about 4.6 S in H_v, HP_v, and E_v, to 5.5 S in Q_v is not known. It appears to depend on the presence of nucleic acid and does not occur in the HP_{av} fractions. A peak with a constant of 4.9 S was observed by Seibert *et al.* (6) in sedimentation experiments with fractions of unheated culture filtrate which had been obtained by electrophoresis so that a mixture of protein and nucleic acid, nearly free of polysaccharide, was obtained. They observed no such peak in the sedimentation of the whole culture filtrate, but this may have been due to the fact that the nucleic acid content of the whole culture filtrate was quite low, 0.5 per cent, as compared to about 5 per cent in our H_v, in which the peak, though small, was observable. There seems to be an increase in sedimentation constant with increasing nucleic acid content in going from E_v to Q_v, and there is a marked change in shape of the sedimentation curve, which approaches, in Q_v, the characteristic curves for nucleic acids. Much light might be thrown on this question if nearly pure nucleic acid, say fraction L, were mixed with different proportions of the protein fractions A and HS¼, and the behavior of the mixtures in sedimentation studied. Lack of sufficient material, among other things, prevents the carrying out of this experiment. Until further work is done with known mixtures of undegraded nucleic acid and proteins, we can observe only that a relatively small amount of nucleic acid seems to have a very large effect on the sedimentation constant of the protein with which it is sedimented.

As to the two proteins for which there is both physical-chemical and serological evidence, one might raise the question, Is one a degradation product of the other? Previous work (3) on the sedimentation and diffusion behavior of proteins isolated from culture filtrates which had been heated 2 and 3 hours and also from an O.T. preparation indicated that the sedimentation constants of the proteins became smaller as the time of heating of the filtrates from which they were obtained increased. Sedimentation experiments on an O.T. pre-

pared from a portion of the H_v culture filtrate, and precipitated four times with 0.5 saturated ammonium sulfate, have shown no indication of material with sedimentation constant higher than 1 S. Indeed no constant can be calculated as the maximum speed of the centrifuge, 60,000 R.P.M., does not supply sufficient driving force to sediment the material at a measurable rate. In none of the heated preparations have sedimentation constants as high as 2.0 or 3.4 S been found. Yet it does not seem possible, in view of the solubility characteristics of HS¼, that it is the precursor of the 2 S protein, the protein in the O.T., or of the proteins studied by McCarter and Watson (3). Our experience with the tendency of HS¼ to precipitate out at room temperature leads us to believe that it would be more likely to precipitate than to break up into smaller molecules when subjected to heat. The fact that no precipitate has been obtained with 0.25 saturated ammonium sulfate in an attempt to fractionate a 2-hour-heated portion of the H_v culture filtrate according to the scheme in Fig. 1 seems to bear this out, although in the serological work, some HS¼ has been found in the 2-hour-heated preparation. The final argument against this relationship is the fact that serologically the HS¼ and 2 S proteins are completely distinct. There is no overlapping in their activities as precipitants as is often the case with native and denatured proteins. It seems probable therefore, that the proteins studied by McCarter and Watson and those present in the O.T. made from H_v culture filtrates are derived by the action of heat on the 2 S protein. It is unfortunate that neither time nor sufficient material has permitted experiments on the effect of heat on fractions such as D, A, or G.

In a recent article Seibert (17) described certain tuberculin proteins referred to as A and B proteins, A being the larger molecule of the two, and B the smaller. It would be convenient to correlate these with the HS¼ and the 2 S proteins, respectively, described here, but this cannot yet be done. The A protein can be identified with HS¼ only if by A is meant TPA-30b described in 1938 (6). The B protein *may* be the same as the 2 S protein although the two definitely differ in certain biological properties; two sedimentation velocity experiments carried out by one of the authors (18) on a sample of this protein sent by Dr. Seibert, showed fairly good peaks, giving a sedimentation constant, s°_{20}, of about 1.8 to 2.1 S, which agrees with what we believe the sedimentation constant of the 2 S protein to be, but until serological experiments prove the two to be the same, or we can obtain some 2 S protein uncontaminated with polysaccharide, no definite statement that they are identical can be made. The reason we prefer to identify HS¼ with TPA-30b rather than with the more recent A protein of Seibert is the following: TPA-30b and HS¼ were isolated in much the same manner, they were both essentially pure protein material, and their sedimentation and diffusion behavior, determined under similar conditions, was the same within the experi-

mental error of the methods. The A protein, on the other hand, is known principally by its electrophoretic behavior, and no data have been published concerning its purity or its sedimentation and diffusion constants.

The third factor discovered in the serological work and called there the "third" antigen, does not appear in the physical-chemical investigations. It is an extremely strong antigen, so that although it is present in all the protein fractions from the virulent strain, except HS¼ and J, it is possibly present in amounts too small to be detected in the sedimentation experiments. This antigen is not present in any fraction from the less virulent strain.

Ammonium sulfate as a fractionating agent leaves much to be desired in the case of tuberculin. If the two proteins could be freed of polysaccharide and yet left unaltered, their separation might then be effected by this reagent. Electrophoresis can do this, but at the expense of time and material.

SUMMARY

Concentrated culture filtrates of two strains of human tubercle bacilli, a virulent and a slightly virulent one, have been fractionated to give fourteen fractions in each case. Chemical determinations and sedimentation velocity measurements have been carried out on those fractions for which significant results could be obtained. The evidence is that two distinct proteins are present, in addition to a polysaccharide and nucleic acid. The physical measurements have not demonstrated the presence of any other proteins. One of the proteins has been isolated in pure form, and found to have a molecular weight of 44,000 ± 5,000, based on measurements of partial specific volume, sedimentation velocity, and diffusion rate. This protein is believed to be the same as one previously isolated by Seibert *et al.* (6), who assigned it a molecular weight of 32,000. The other protein was not obtained sufficiently free from polysaccharide so that its molecular weight could be determined, but it is believed to have a sedimentation constant of about 2 S. Sedimentation and diffusion constants have been obtained for the polysaccharide, which appears to be a homogeneous molecular species with a molecular weight of about 20,000. The source in unheated tuberculin of the proteins obtained from heated preparations is discussed.

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THE PROTEINS IN UNHEATED CULTURE FILTRATES OF HUMAN TUBERCLE BACILLI

II. Determination of Serological Properties

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INTRODUCTION

Preparations obtained from unheated culture filtrates of tubercle bacilli by fractionation with ammonium sulfate were sufficiently distinctive according to their sedimentation and diffusion diagrams (1) to warrant a detailed quantitative investigation of their serological identities. Qualitative tests had previously indicated some differences in the precipitinogenic activities of purified protein derivative (P.P.D.) tuberculins (2) prepared in different ways, and these variations could be correlated with different degrees of activity in eliciting skin reactions in human beings (3). It was our hope that a correlation of the serological and the physicochemical data would indicate, first, the number of proteins in unheated culture filtrates and, second, whether or not skin activity resided in any particular kind or condition of protein.

By means of quantitative precipitin and precipitin absorption tests two distinct antigens were found among the protein fractions separated from the culture filtrates of both fully virulent and slightly virulent human tubercle bacilli: the presence in the various fractions of these two antigens could be correlated with the appearance on the sedimentation diagrams of components with constants of about 3.4 S and of about 2 S (4). The material giving the higher rate was in a fraction precipitated by 0.25 saturated ammonium sulfate at pH 4.4 which was practically homogeneous antigenically. The fractions obtained by full saturation with ammonium sulfate contained only the second serological entity. Fractions obtained by 0.5 and 0.75 saturation were mixtures of the two, according to both precipitin tests and sedimentation diagrams. A third antigen, and the one most active in eliciting antibody formation, was found only in the fractions from the virulent culture filtrate. The chemical and physical nature of this antigen is unknown since we have demonstrated its presence only by serological means.

That the two protein antigens were entities was shown also by another type of serological test; namely, the passive transfer of skin sensitivity to normal guinea pigs by the serum of guinea pigs sensitized to our protein fractions (4).

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All fractions proved to have skin activity for tuberculin-sensitive human beings or tuberculous guinea pigs. Thus this property cannot be associated with one or the other of the two protein antigens. With the antigenically homogeneous fractions it became possible to demonstrate that the subject tested rather than the kind of protein is responsible for certain differences in the nature of the skin reactions in human beings. To cite an example, those persons who started to react almost immediately upon the injection of a tuberculin responded early to either of the antigens. Also, a tuberculin-sensitive individual, whether he had active tuberculosis, or calcified lesions only in his lungs, or no signs of tuberculosis upon chest x-ray, reacted alike to the serologically distinct fractions.

The two protein antigens both proved to be capable of sensitizing the skin of normal guinea pigs (4): the sensitizing doses were of the order of magnitude of those commonly used in skin testing tuberculous guinea pigs. However, the sensitization was against only the homologous fraction. The skin reactions differed somewhat from those in tuberculous guinea pigs in certain characteristics, the significance of which is not known. The results leave it an issue as to whether or not the fundamental mechanism is the same for infected and protein-sensitized animals. Possibly a tuberculous animal gives a skin reaction only to a protein against which it has been specifically sensitized: if this is so, tuberculous human beings who reacted to the two protein antigens must have been specifically sensitized by both of them.

EXPERIMENTAL

Preparation of Fractions.—Culture filtrates were prepared both from a virulent (DT[1]) and from a slightly virulent (TB–1[1]) strain of human tubercle bacillus. The particular cultures of the virulent strain used for seeding were the second subcultures from ones shown to have full virulence for guinea pigs. The slightly virulent strain in doses of 1 mg. inoculated subcutaneously in finely divided suspensions produced tubercles in guinea pigs but the animals were still living after a year. This culture can be identified as a tubercle bacillus from its history of original virulence, from the fact that it had been successfully used during some 20 years to make the tuberculin for state cattle testing, and from the cultural characteristics of rate of growth and growth temperature requirements under given conditions.

The following synthetic medium (Henley (5)) was used to grow the organisms:

$K_2HPO_4 \cdot 7H_2O$	50 gm.
Sodium citrate, $5\frac{1}{2}H_2O$	25 "
$MgSO_4 \cdot 7H_2O$	40 "
Glycerine	3,500 cc.
Ferric citrate	3.2 gm.
Asparagine	250 "

The ingredients were put into solution and added in order named and the volume made up to 50 liters. The pH of the medium before autoclaving was 7.0. The cultures were incubated

[1] The DT culture came from the Bureau of Animal Industry, and was kindly sent to us by Dr. Florence Seibert. The culture TB–1 also originally came from the Bureau of Animal Industry.

at 37°C. and held at this temperature for varying lengths of time after marked autolysis had taken place. To determine the effect of length of autolysis upon the protein content of the filtrates, two lots of virulent cultures were set up at different times and one held for a total incubation of 9 weeks and the other for 21 weeks. The cultures of the slightly virulent strain were held 9 weeks before harvesting. For investigating the effect of heat upon the protein content of the filtrates, cultures from the lot held 21 weeks were treated in three different ways. One part, the cultures from 15 of the original 50 liters of medium, was made as an old tuberculin (O.T.); *i.e.*, the cultures were held for 4 hours in a steamer with the liquid about 100°C., the cells filtered off through paper, and the filtrate evaporated on a steam bath with a jet of air blowing over the surface, to one-tenth the original volume of the culture fluid. A second part of the cultures from 6.5 liters of original medium was held in the steamer for 2 hours (the method used recently for making P.P.D. tuberculins (2)) and allowed to cool in air to room temperature. The third part of the culture filtrate originally representing 24 liters was not heated; after filtration to remove the cells it was held at 5°C.

Cellular material was removed by filtration through paper, then coarse and finally fine Mandler filters. The filtrates were immediately concentrated and washed by ultrafiltration with phosphate buffer (0.005 M, pH 7.4 to 7.5) at 5°C. (2). Throughout the process merthiolate was maintained at a concentration of 1/10,000.

To ascertain whether we might be losing any significant constituents by ultrafiltration, parts of the ultrafiltrates were in turn concentrated and washed in the alundum cups impregnated with heavier cellulose acetate films. To prevent any changes which might occur upon standing in solution, all preparations were lyophilized immediately after they had been washed by ultrafiltration.

Fractionation was carried out, as previously described (1), on the preparation from the 21-week virulent culture filtrate and on that from the 9-week slightly virulent culture filtrate. This consisted essentially in a preliminary partition with acetate buffer at pH 4.4, and a fractionation of both acid supernatant and precipitate with ammonium sulfate to give successively precipitates at 0.25, 0.5, 0.75, and full saturation.[2] Estimations of protein, polysaccharide, and nucleic acid contents were made on each fraction as previously described. These fractions were used for detailed serological investigation.

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[2] The fractions will be given here the same designations as in the first paper of this series (see Fig. 1 (1)). The following fractions were obtained from the acid supernatant:
from the part precipitated in a preliminary treatment with 0.5 saturated ammonium sulfate at pH 7.2

B by treatment with acetate buffer at pH 4.4
HS¼ by 0.25 saturation with ammonium sulfate at pH 4.4
G " 0.5 " " " " " " 4,4
K " 1.0 " " " " " " 4.4

from the part soluble in 0.5 saturated ammonium sulfate

A by 0.75 saturation with ammonium sulfate at pH 7.2
J " 1.0 " " " " " " 4.4
M soluble in 1.0 saturation with ammonium sulfate at pH 4.4.

The other fractions were precipitated from the acid precipitate as follows:—

E by 0.25 saturation with ammonium sulfate at pH 7.2
N " 0.5 " " " " " " "
P " 0.75 " " " " " " "
Q " 1.0 " " " " " " 4.4.

The subscript "v" identifies the fractions from the virulent culture filtrate and "av" those from the slightly virulent.

Antiserum Preparation.—Antisera were produced against certain of the fractions by the intracutaneous injection into rabbits of the materials at 3 day intervals to a total of approximately 100 mg. of protein per animal.

Specific Precipitation.—The fractions were characterized as to their antigens by determining the amounts of specific precipitate nitrogen with the procedures of Heidelberger and Kendall (6) and Heidelberger, Kendall, and Soo Hoo (7). All precipitations and operations were carried out at 0–5°C. The amounts of fraction protein nitrogen used as precipitants were the smallest quantities which would give maximal precipitates as based on preliminary tests; one-half these amounts of antigen were used for all succeeding absorptions. The first precipitates were taken out after 48 hours and subsequent absorptions were each held for 1 week. All antisera had been tested for polysaccharide antibodies with the carbohydrate we had isolated from the virulent culture filtrate (fraction M) and with one of those obtained from tubercle bacillus cells[3] by Heidelberger and Menzel (8). A few sera showed very slight precipitates with polysaccharides; they were preliminarily absorbed with the cell polysaccharide, 5 μg. per cc. of antiserum: this quantity was sufficient to absorb the polysaccharide antibodies but small enough not to cause precipitation of protein antibodies by the contaminating protein.

RESULTS

When the fractions from the unheated virulent and slightly virulent culture filtrates were tested with an antiserum against the unfractionated unheated virulent culture filtrate, at least three serological entities were found. The results are shown in Table I. The combined quantitative and absorption data show that the J_{av} and the HS¼[4] fractions are distinct and that the P_v fraction contains at least one other antigen.

Further evidence that the isolated fractions HS¼ and J_{av} are different antigenically is provided in the data in Table II. The use of the O.T. antiserum as a source of antibodies for the "saturated" antigen[5] was justified by the following considerations, and thus in the preparation of the antiserum inroads did not have to be made into the small yields of the J fractions. The O.T. contained none of the 0.25 antigen[5] as even large amounts failed to cause precipitation in the HS¼ antiserum. The small amount of precipitate obtained with the J_{av} fraction in the HS¼ antiserum can be explained by assuming a slight admixture of the "saturated" protein in the HS¼ preparations: this has been later corroborated by other types of biological tests. This would explain why relatively large amounts of HS¼ fractions brought down precipitates in the O.T. serum while small quantities did not, and also why long continued absorptions with HS¼ fractions will eventually exhaust the O.T. antiserum of precipitins for the J fractions.

[3] Their label "$520B_2^{17}$." From their figures this polysaccharide contained 3.25 per cent nitrogen.

[4] Since the yield of the HS¼ fractions was so small, and since the sedimentation pictures and preliminary precipitin tests indicated the proteins were similar, the v and av fractions were combined and are referred to as HS¼.

[5] The antigen present in the HS¼ fractions will be referred to as the 0.25 antigen, that in the J fractions as the "saturated," and the other one present in the P_v fraction (Table I) will be called the third antigen.

The much greater antibody-eliciting potency of the third antigen present in the P_v fraction (Table I) and the further differentiation of this antigen from the 0.25 and the "saturated" antigens are indicated in Table III. The large amount of antibody for this third antigen in the serum described in the table made possible its detection without the use of absorbed sera or quantitative tests, but the quantitative data show strikingly its relative antigenic power.

TABLE I

Amounts of Precipitates Formed When Three Different Protein Fractions Were Added to an Antiserum Prepared by Injecting Unheated Unfractionated Ultrafiltered Culture Filtrate of Virulent Tubercle Bacillus

Antiserum*	Fraction used as precipitant	No. of absorption	Precipitant protein N added per cc. antiserum	N precipitated per cc. antiserum
			μg.	μg.
Unabsorbed	P_v	1st	100	210
		2nd	50	60
		3rd	50	30
		4th	50	30
		5th	50	0
"	J_{av}	1st	80	40
		2nd	40	+‡
Absorbed with J_{av}	HS¼	1st	180	40
		2nd	90	+
Absorbed with J_{av} and then with HS¼	P_v	1st	10–100	Significant amount of precipitate not determined quantitatively

* For absorption with the P_v fraction 6 cc. of antiserum were used and with the J_{av}, 5 cc.
‡ Less than 10 μg.

The data thus reveal the presence of a major antigen in the HS¼ fractions, another in the J_{av} fraction, a combination of these two in the A_v fraction, and a third antigen in the A_v and P_v fractions from the virulent culture filtrate.

To assay all the fractions from the virulent and slightly virulent culture filtrates, three antisera were used; namely, (1) to test for 0.25 antigen, antiserum to HS¼ with small amount of antibody to "saturated" antigen absorbed out with J_{av}; (2) to test for "saturated" antigen, antiserum to A_{av}, absorbed with HS¼; (3) to test for the third antigen, antiserum to unheated unfractionated virulent culture filtrate, absorbed with HS¼ and with J_{av}.

The results presented in Table IV indicate that in general fractions obtained by 0.25 saturation with ammonium sulfate contained the 0.25 antigen, likewise those precipitated by saturated ammonium sulfate had the "saturated"

TABLE II

Amounts of Precipitates Formed in Homologous and Cross-Reactions of 0.25 and "Saturated" Antigens in Their Antisera

Substance used to produce antiserum	Substance used to absorb antiserum	Substance used as precipitant	No. of absorption	Precipitant protein N added per cc. antiserum*	N precipitated per cc. antiserum
				μg.	μg.
HS¼		HS¼	1st	10	90
			2nd	5	10
			3rd	5	0
			4th	8	+
			5th	8	+
"		J_{av}	1st	1‡	10
			2nd	0.5	0
			3rd	0.5	0
			4th	0.5	0
"		O.T.$_v$ unfractionated		160§	0
				16§	0
O.T.$_v$ unfractionated		J_{av}	1st	30	30
			2nd	15	+
			3rd	15	+
" "		HS¼	1st	90	20
			2nd	45	+
			3rd	45	+
			4th	45	+
			5th	45	0
" "	J_{av}	HS¼		80	0
" "	HS¼ 3 times	J_{av}		5	++‖
" "	HS¼ 5 times	J_{av}		5–50	0

* For each set of absorptions 3 cc. of each antiserum were used.

‡ Larger ratios of antigen to antibody gave no precipitate and were undoubtedly in the antigen excess zone.

§ Nitrogen of portion precipitable by alum.

‖ Not determined quantitatively.

antigen, and those obtained by 0.5 or 0.75 saturation had both antigens. The third antigen was present in all the preparations from the virulent culture filtrate except in HS¼ and in J. Thus J_v and J_{av} have the same antigenic constitution, as well as HS¼$_v$ and HS¼$_{av}$.

TABLE III

Comparative Amounts of Precipitates Formed by the Three Antigens in an Antiserum against Fraction A_v

Fraction used as precipitant	Precipitant protein N added per cc. antiserum	N precipitated per cc. antiserum
	μg.	μg.
HS¼	80	45
J_{av}	80	++*
P_v	160	730
A_v	160	740

* Not determined quantitatively but approximately same visible amount of precipitate as yielded by HS¼.

TABLE IV

The Distribution of the Three Antigens in the Fractions from the Virulent and the Slightly Virulent Culture Filtrates

Fraction used as precipitant*	Antiserum against		
	0.25 antigen	"Saturated" antigen	Third antigen
$HS¼_v$	+	0	0
G_v	+	+	+
A_v	+	+	+
J_v	0	+	0
K_v	0	+	+
E_v	+	0	+
N_v	+	+	+
P_v	+	+	+
Q_v	0	0	+
G_{av}	+	+	0
A_{av}	+	+	0
J_{av}	0	+	0
N_{av}	+	+	0
P_{av}	+	+	0

* For each individual test quantities corresponding to 0.15 cc. of undiluted antiserum were used. The amount of fraction protein used as antigen varied with the particular antiserum: in general it was twice as much of the fraction protein to be tested as of the homologous fraction which would give maximal precipitate with the antiserum. This quantity was always considerably less than the amount of homologous fraction which would be in the antigen excess zone. For the 0.25 antibody the ratio was 300 μg. of fraction protein per cc. of serum; for the saturated antiserum 500 μg. of protein; and for the "third" antibody 100 μg. of protein. Negative results were checked with a range of dilutions of each fraction.

The individuality and the antigenicity of the 0.25 and "saturated" antigens have been demonstrated also by skin tests on both actively and passively sensitized guinea pigs. For active sensitization J_v was injected into seven guinea pigs. Fifty μg. doses of protein were given intracutaneously on the belly in 0.1 cc. volumes at 5- to 6-week intervals. Small but definite skin reactions appeared in response to the second injections and the inflammation elicited increased markedly up through the fourth injections. When it was judged that the animals would give maximal skin reactions, they were tested with the sensitizing fraction and, 72 hours later, with one heterologous antigen. To control the effect of one injection on the succeeding injection, the tests were repeated. An animal thus received four injections, two of homologous and two of a heterologous fraction, with 72 hours between each injection. The solutions were put in intracutaneously on the flank and as usual in a volume of 0.1 cc. Reactions in the skin of the flank have better definition than those in the abdominal skin. Only one fraction was given at a time since previous work on animals sensitized with heat-killed tubercle bacilli had indicated that the skin reaction to one fraction might be inhibited by the simultaneous injection of another fraction. The reactions to two equal doses of the same fraction given 144 hours apart were of approximately the same intensity and therefore the method of comparing fractions by injecting them 72 hours apart was deemed sufficiently accurate.

TABLE V

Specificity of Skin Reactions in Guinea Pigs Actively Sensitized with Fraction J_v When Tested with the 0.25 and "Saturated" Antigens

Animal No.	Test fraction	Amount of test fraction protein injected	No. of previous injections of J_v	Skin reactions after 18 hrs.*
		μg.		mm.
1	J_v	10	Six	21 × 21 (× 2)
	B_v	20		7 × 7 (× 1)
	J_v	2		9 × 9 (× 1.5)
2	B_v	20	Three	10 × 10 (× 1)
	J_v	20		28 × 26 (× 2.5)
3	M_v	1,000 (polysaccharide)‡	Four	22 × 22 (× 2.5)
	J_v	50		23 × 23 (× 3)

* The dimension in parentheses is the height of the inflamed area estimated for comparative purposes by measuring the thickness of the doubled skin at the center of the reaction and dividing by two.

‡ Estimated from N content to contain about 50 μg. of protein.

Typical results appear in Table V.[6] All the skin reactions started almost immediately with erythema, edema, and slight purplish discoloration at the center. They reached their height at 18 to 19 hours when they showed slight erythema, rather firm edema, and lavender to reddish purple centers not over 3 mm. in diameter. By 48 hours the reactions had almost disappeared. (How-

[6] Note that B_v was used in place of HS¼ since our supplies of the latter fractions were insufficient. Serologically the B fractions were similar to HS¼ and physical chemical data indicated the B fractions to be slightly denatured HS¼.

ever, it should be noted here that a stronger reaction was obtained in one guinea pig sensitized with B_v in the same way as the J_v animals. In this particular animal 100 μg. were given for the fourth sensitizing dose, and the reaction was still marked, although decreasing, at 48 hours, and had an ulcerated center 10 mm. in diameter.) The reactions varied in general from those seen in animals sensitized with tubercle bacilli in that the amount of central necrosis was relatively small compared to the volume of the edema, although reactions have been seen in guinea pigs sensitized with heat-killed tubercle bacilli which reached maximal size at 24 hours. At that time also the latter reactions could not be distinguished by their other characteristics from simultaneous reactions in the animals sensitized with the J_v fraction.

The primary injection of a fraction into the normal animal served as a control on the toxicity of the substance and on the condition of the guinea pig with respect to tuberculous infection. The usual response was an almost colorless edema, not over 10 mm. in diameter, which appeared almost immediately and lasted for only 6 to 8 hours. One animal was regarded as suspicious because it gave a primary reaction of slight, almost colorless edema 15 mm. in diameter at 18 hours. The subsequent reactions of this animal gave no indications that it was tuberculous and the conclusion was drawn that the toxic action of J_v varies slightly with the animal.

For making serological tests small amounts of serum were taken from each J_v-sensitized animal and from animals sensitized similarly with the fractions J_{av}, G_v, G_{av}, and B_v. Trial bleedings were made at various intervals but the most potent sera were obtained when the blood was taken about a week after an injection which had resulted in a maximal skin reaction. Precipitins could not be demonstrated in these sera but the presence of antibodies was made manifest by the moderately large skin reactions obtained when the sera were injected into the skin over the flanks of normal guinea pigs and the fractions injected 48 hours later. This type of reaction has been shown by Chase (9) to be given by guinea pigs injected with the sera from rabbits and guinea p gs sensitized with various proteins such as horse serum, and has been fully described more recently by the same author (10). The details of some of these passive transfer reactions with the sera of sensitized guinea pigs are given in Table VI.

Quantities of 0.1 or 0.15 cc. of the undiluted antiserum were injected into the skin of normal guinea pigs in the weight range of 250 to 300 gm. Animals of this size have been found to give optimal skin reactions of this type. When the fractions were given subcutaneously they were put over the belly and when intracutaneously they were put in locally as closely as possible to the site of injection of the antiserum. Where injections of two different fractions were given successively, the interval between was that denoted in the table as the time when the reaction to the first injection was negative. In the case of the last animal in Table VI the injections of the two antisera were made in the same animal and the reactions were simultaneous.

The data in Tables V and VI again indicate that the 0.25 and the "saturated" antigens are distinct, and that both have about the same potency as antibody-eliciting substances. The animals actively sensitized with the J fractions gave only slight skin reactions to the B_v fraction and the serum from the J-

TABLE VI

Skin Tests Showing Lack of Cross-Reactions to Fractions in Passively Sensitized Guinea Pigs

No. of guinea pig donor	Fraction used to sensitize donor	Test guinea pig No.	Test fraction	Test fraction protein	Route of injection*	Skin reaction	
						Time when maximal	Size in mm. and color‡
				μg.		hrs.	
4	J_{av}	40	J_{av}	30	sc	0.42	42 × 37 vfp− (++)
4	J_{av}	41	B_v	30	"	2	0
			J_{av}	30	"	0.5	27 × 21 vfp (+)
5	J_v	50	J_v	30	"	0.25	31 × 31 vfp+ (+++)
5	J_v	51	B_v	30	"	1.17	0
			J_v	30	"	0.28	32 × 32 vfp− (+)
6	B_v	60	B_v	30	"	0.58	57 × 38 fp (+++)
6	B_v	61	J_{av}	30	"	0.3	22 × 22 fp (±)
6	B_v	62	J_v	30	"	19	0
			B_v	10	"	3.5	45 × 35 alcls (++)
7	G_v	70	J_{av}	10	ic	3	18 × 17 vfp (+++)
7	G_v	71	B_v	10	"	0.2	25 × 14 vfp (++)
7	G_v	72	G_v	100	sc	0.42	33 × 29 vfp− (+)
8	G_{av}	72	"	"	"	"	35 × 30 vfp− (+)

*sc = subcutaneously; ic = intracutaneously.

‡ The number of +'s in parentheses is an estimation of the relative height, since measurement of height would have disturbed reaction. v = very; f = faint; p = pink; al = almost; cls = colorless.

sensitized guinea pigs transferred no sensitivity to the B_v fraction. Conversely the serum from the animal sensitized with the B_v fraction transferred to one animal a very slight sensitivity to the J fraction. Thus the data gave corroboration that the HS¼ has a small admixture of the "saturated" antigen. The G fractions were further shown to have both the 0.25 and the "saturated" antigens. The serum from the G_v- and that from the G_{av}-sensitized animal

transferred about the same degree of sensitivity to the G_v fraction. That is, the third antigen had not been effective in producing antibodies in the G_v guinea pig probably because such small amounts were given. From the tests in the actively sensitized animal the protein in the M_v fraction, the one made up almost entirely of polysaccharide, appeared to be the same as that in the J_v fraction.

DISCUSSION

A close correlation exists between the results of the serological investigations and the sedimentation studies reported in the previous paper (1) in demonstrating the presence of the 0.25 and the "saturated" antigens in the various fractions separated from the filtrates of both the virulent and the slightly virulent cultures. However, the precipitin test is the more sensitive method, since it revealed the presence of two proteins in the G_{av} fraction which upon sedimentation was not resolved into two peaks. (The peaks for both the 3.3 S and the 2 S proteins do appear in the sedimentation diagrams of G_v, A_v, and A_{av}.)

These two antigens are proteins according to their behavior in sedimentation and in specific precipitation: that is, the mass of a fraction being sedimented as determined from the area under the curve and the quantity of the fraction necessary to bring down a given amount of specific precipitate N can both be correlated with chemically determined protein content. Although certain of the fractions, notably J_v and J_{av}, contain considerable percentages of polysaccharide, nevertheless the evidence is that the antisera prepared against the fractions are not directed to any measurable extent against their polysaccharide moiety. Thus, although the polysaccharide in the fractions will react in the horse antiserum (No. 5807L) (8) made against dead tubercle bacilli, the rabbit and guinea pig antisera prepared by injecting the fractions do not react with the polysaccharide in fraction M or with a polysaccharide from dead tubercle bacilli.

The two antigens are probably uniformly present in unheated culture filtrates of human tubercle bacilli: we have demonstrated their presence in 9- and 21-week old culture filtrates of a virulent strain and in the 9-week filtrate of a slightly virulent one. The HS¼ fractions from the three different filtrates were shown to be the same in sedimentation behavior in the ultracentrifuge, and to be the same as the one isolated by Seibert *et al.* (11).

The third antigen was found by means of the serological tests in both the 9-week and the 21-week culture filtrates of the virulent strain. Its nature and significance are not known. No evidence of its existence is found on the sedimentation diagrams of any of the fractions. Its presence is not correlated with the nucleic acid content of the fractions: although it is present in culture filtrates high in nucleic acid, it is absent from the J_v fraction which contains a large percentage of nucleic acid. It is probably present in very small quantities

in the fractions as relatively large amounts of the latter were required to bring about precipitation of the ant body to this antigen; furthermore, it did not sensitize the guinea pig injected with small quantities of the G_v fraction. This third antigen can cause errors in testing for the two known protein antigens unless its antibody has been demonstrated not to be present in the serum being examined.

These three antigens appear to be the major ones of unheated tubercle bacillus culture filtrates. Serological attempts to demonstrate others in either the crude preparations or the fractions were unsuccessful. The ultrafiltrates from the ultrafiltered culture filtrates when in turn concentrated by ultrafiltration had very weak skin activity for tuberculous guinea pigs and were therefore not studied further.

The HS¼ and the J fractions give a typical tuberculin type of skin reaction. This would seem to be the reason why O.T. can be used for intracutaneous testing even though the HS¼ protein is labile to heat. In cultures heated for 2 hours the 0.25 antigen could be demonstrated in two ways: the antiserum against the ultrafiltered but unfractionated culture filtrate contained antibodies for HS¼ and the same heated culture filtrate was precipitated by the antiserum against HS¼. The "saturated" antigen was also present and in much larger percentage in the 2-hour-heated preparations. In the culture filtrates heated for much longer periods to make a true O.T., however, the 0.25 antigen was absent although the "saturated" antigen remained and could still stimulate antibody formation. Undoubtedly much of the 2 S protein is changed markedly during the heating process because the material remaining in solution when the O.T. was fractionated with acetate buffer at pH 4.8 could not be sedimented at a measurable rate in the Svedberg u tracentrifuge. The acid precipitate was inhomogeneous upon ultracentrifugation. Thus the remaining protein must be denatured, but neverthe.ess some of the 2 S protein remained active and recognizable antigenically. (The third antigen was also heat-stable as its presence was determined serologically in the O.T.)

It is possible that the protein remaining in o d tuberculins is denatured "saturated" or 2 S protein most of which is partially degraded. A fraction separated by Seibert from an O.T. was barely resolvable in the ultracentrifuge and proved to be relatively homogeneous with a constant of 1 S (3).[7]

The skin activity of the 1 S protein separated by Seibert from O.T. is quantitatively different from that of a fraction separated from a 2-hour-heated culture filtrate (the filtrate in this case being different from the one used to make the O.T., however)(3). When our O.T., 2-hour-heated tuberculin, and unheated culture filtrate, all concentrated and washed by ultrafiltration and all originating from the same culture filtrate, are compared on a nitrogen basis in skin

[7] P.P.D. 67–2.

tests on tuberculous guinea pigs, the unheated material is slightly stronger than the 2 hour-heated preparation and both are much more potent than the O.T. or even than the most active protein fraction made from the O.T. The greater potency of the unheated or 2-hour-heated proteins is not a desirable feature in a practical testing agent since all actively tuberculous human beings will react to the 1 S O.T. preparation (3). The unheated proteins have the disadvantage moreover of sensitizing the skin when given in very small doses whereas the 1 S protein is not antigenic (3).

As to the nature of the active skin agent in tuberculins, we must conclude that two completely different but equally potent proteins are present in the culture filtrate, or that the 3.4 S protein and the 2 S protein have a common skin-reactive grouping which does not influence the antigenic specificity of either one, or that some active substance is precipitated equally with both proteins.

The HS¼ fraction is shown to be a good precipitant in comparison with other known bacterial proteins: with 10 μg. of protein N 90 μg. of N were precipitated from 1 cc. of antiserum. The third unknown antigen is the best known precipitinogen among tuberculin or tubercle bacillus constituents in that about 700 μg. of specific precipitate N was brought down by about 150 μg. of fraction protein N so that more than 550 μg. of precipitate N must have come from the antibody.

SUMMARY

1. Only two serologically different proteins were found in the unheated culture filtrates of both virulent and slightly virulent tubercle bacilli. One of them was the protein which had a sedimentation constant of 3.4 S, and the other was in filtrate fractions with a constant of 2 S.

2. That these proteins were distinct was demonstrated by three methods: quantitative precipitin and precipitin absorption tests with rabbit antisera, skin tests in guinea pigs actively sensitized with the culture filtrate fractions, and skin tests in passively sensitized guinea pigs.

3. A third antigen of unknown nature was found by means of the precipitin tests, but only in certain fractions from the virulent culture filtrate.

4. The protein with the constant of 3.4 S could not be demonstrated serologically in an O.T. made from the same culture filtrate as the unheated preparation from the virulent organism.

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STUDIES
FROM
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Announcement

Volumes 131 and 132 of the *Studies from The Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

A STUDY OF NERVE PHYSIOLOGY

The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1060 pages with 480 illustrations.

In order that the volumes may be available to those who do not receive them as part of their subscriptions to the *Studies*, extra copies have been printed which are purchasable at the regular price of $2.00 per volume. The two volumes will only be sold together. They can be obtained from the Publication Service, The Rockefeller Institute for Medical Research, York Avenue and 66th Street, New York 21, N. Y., at a price of $4.00, payable in advance.

CHEMICAL STUDIES IN HOST-VIRUS INTERACTIONS*

IV. A Method of Determining Nutritional Requirements for Bacterial Virus Multiplication

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Many studies with multicellular organisms have shown that the multiplication of virus depends on the nutrition of the organism. A certain dependency was to have been expected, but the specific character of this dependency has proven unpredictable in many cases. For example, thiamine deficiency diminishes the susceptibility of mice to some strains of poliomyelitis virus (1), but on the other hand lowers the resistance of pigeons to psittacosis (2). A study of these specificatio ns may be expected to provide important clues to the mechanism and control of a particular viral infection.

The purpose of the present studies has been the investigation of methods by which nutritional requirements can be detected. The knowledge that a metabolite is required suggests the use of an antimetabolite which will interfere with the process. This thesis has been well developed by Wooley (3) and others, and provides a rational approach for chemotherapeutic investigation. In the present series of studies, the effect of two antimetabolites specific for determined requirements has been shown to interfere with virus multiplication, illustrating again the validity of this approach. Some studies of the effects of organic components of a medium on bacteriophage production have already been made by Wahl (4) and Spizizen (5, 6). These studies, in addition to the previous experience in this laboratory, made it expedient to use the technically favorable and uncomplicated *Escherichia coli*-T2 bacteriophage system.

Evidence of the dependence of virus synthesis on the constituents of the medium of the host cell has been presented (7). It has been shown that *E. coli* infected with T2 virus assimilate the nitrogen and phosphorus of the simple medium F at a considerable rate. The metabolism of phosphorus is directed exclusively to the production of the phosphorus-containing compound of T2, desoxyribose nucleic acid. Using radioactive phosphorus, it was also shown that most of the phosphorus in the synthesized virus is derived from the medium

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after infection. Other studies (7) including those described below indicate that similar relationships prevail for the utilization of the carbon and nitrogen of the medium.

It has been shown previously (8) that *E. coli* do not multiply after infection. Therefore, it is considered, pragmatically, that those compounds found to increase the number of virus particles produced or rate of production are those which are used in virus synthesis. This does not preclude a rapid organization or turnover by the bacterial cell of the material which, under normal conditions, might be used for its own growth and multiplication.

Two methods of determining whether a specific compound is involved in virus synthesis have been previously described. First, analysis of the T2 virus showed desoxyribose nucleic acid, arginine, tryptophane, and tyrosine to be virus constituents (8). A more complete analysis may be presumed to give a wider scope to the choice of an antimetabolite. Second, an antimetabolite was tested for inhibition of virus synthesis. Reversal of the inhibition with the specific metabolite indicates that it is required for synthesis (9). In the course of studies with 5-methyl tryptophane, a specific antagonist for tryptophane utilization (9), two interesting effects were observed in addition to the confirmation that tryptophane was essential for virus synthesis. First, it was shown that it is possible to determine at what time during the latent period of multiplication within a host cell a compound is required, and secondly, infectious centers were irreversibly destroyed by the action of the compound after a certain period of inhibition of virus multiplication.

This paper presents a third method of determining whether a specific compound is required for virus synthesis; *i.e.*, the effect of addition of a specific compound to the system in a condition suboptimal for virus synthesis. The times required for multiplication of T2 virus within a host cell are almost the same if the cell is grown and infected in either a complex medium such as nutrient broth or in a simple synthetic medium containing glucose (10) or lactate (9) as a carbon source. This does not mean, however, that the latent period is independent of the nutrients present in the medium, since these systems contain two independent and compensating variables. It is possible that bacteria grown in the absence of external amino acids have greater abilities to synthesize these compounds than bacteria grown in a complex medium, such as broth. That the latent periods for these two systems are identical may mean that the rates at which components of virus are made available for organization, in the one case by synthesis, in the other by absorption, are identical.

When the multiplication of virus is followed in a system with just one variable, *i.e.* the composition of the medium, the dependence of virus synthesis on the composition of the medium is readily observed. *Escherichia coli* grown in broth and transferred and infected in a simple synthetic medium show a much longer latent period and a much smaller burst size than the same bacteria in-

fected in broth. The simple medium may be supplemented with various compounds. A study of the time required to liberate virus and of the number of virus particles synthesized, as compared to the unsupplemented medium, indicates whether or not the particular compound is a rate-determining factor in virus synthesis under these conditions. Many substances were found to stimulate synthesis, but no single supplementary compound was found which approximated the rate and amount of virus synthesis characteristic of broth.

It has been proposed (11, 12) that virus synthesis is the autocatalytic conversion of preformed cellular protein into virus. If this were the case, the number of steps in the process of synthesizing active virus from inactive precursor would be expected to be very few and to involve the specificities of large molecules. The control of this type of process might be very difficult. We have found, however, that synthesis of virus depends on the presence of simple compounds in the external environment of the host cell. It would seem that a great many syntheses of relatively simple compounds are essential stages in virus multiplication. Such a process should be susceptible to inhibition by a wide variety of antimetabolites.

Materials and Methods

Escherichia coli B was subcultured monthly to Difco agar nutrient broth slants. From these, weekly subcultures were made. Cultures were made by inoculating broth medium, containing 8 gm. of Difco nutrient broth and 5 gm. of NaCl per liter of distilled water, with bacteria from a slant and aerating at 37°, overnight. At the end of this time, the culture contained about 5×10^9 viable bacteria per cc. 0.05 cc. was transferred to 10 cc. of fresh broth and grown to about 5×10^7 bacteria per cc. with aeration at 37°. The broth cultures were then centrifuged, washed twice in a simple defined (8) medium (F), and resuspended in F, F plus a supplement, and N. Bacteria treated in this way were designated B_N-F, and B_N-N, the subscript indicating the medium in which the bacteria were grown and the large letter the medium in which they were resuspended. The resuspended bacteria were infected with a purified T2r$^+$ concentrate prepared from F medium or broth lysates (13, 14). All T2 virus referred to in this paper was the r$^+$ type. The liberation of virus in the same host cells in different media was followed by the one-step growth technique of Delbrück and Luria (15) using the spreading and layer modification of Hershey *et al.* (16).

EXPERIMENTAL

Bacteria grown in N and infected in N (B_N-N) liberate virus consistently at 20 to 22 minutes with a burst size or yield of virus particles per infected cell of from 80 to 200. The same bacteria resuspended in F (B_N-F) do not start liberating virus until at least 40 minutes and in some cases as late as 70 minutes after infection. The burst size in F has been found to vary from 5 to 30. An experiment of this type is presented in Fig. 1.

Other evidence that B_N-F are deficient in their synthetic capacities is obtained from studying the turbidity curves of B_N grown in N, washed, and resuspended in N and F. Fig. 2 shows that B_N in F has a much slower growth rate than the same bacteria in broth.

The effect of the different constituents in F medium was determined by preparing a series of F media each lacking a single constituent. The total concentration of all other ions was kept approximately constant by the addition of appropriate salts. Table I gives the details of this experiment.

It may be seen in Fig. 3 that an external source of nitrogen and carbon is essential for virus synthesis. Virus liberation and yield seem to be almost inde-

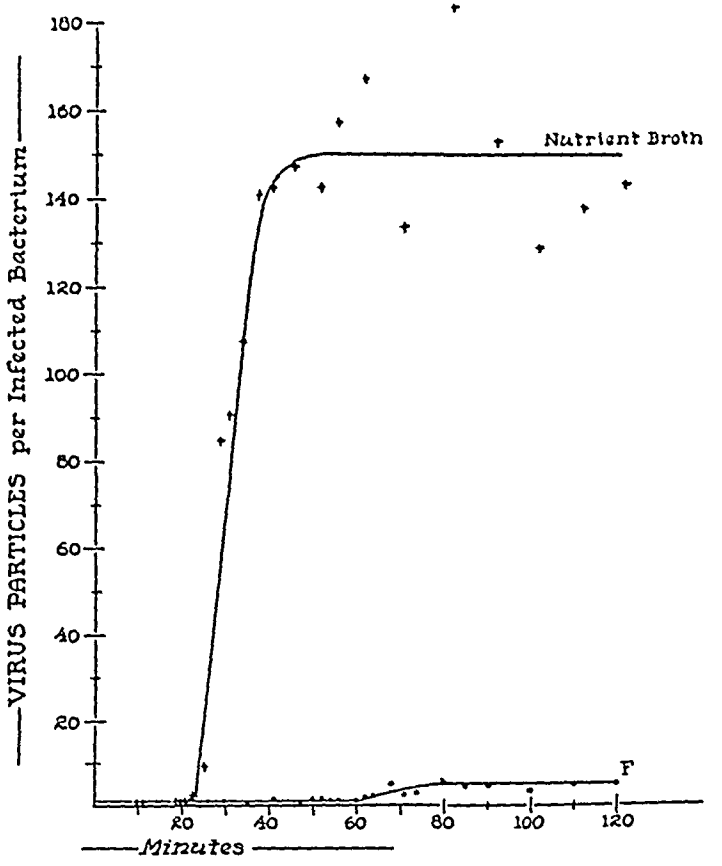

FIG. 1. One-step growth curves for broth-grown bacteria in the broth and F media.

pendent of added sulfate. The absence of external phosphate severely limited virus multiplication, decreasing the yield of virus and significantly increasing the latent period. The absence of magnesium was also found to limit virus synthesis.

In connection with the apparent lack of a sulfur requirement, it must be noted that the other constituents of the medium may be sufficiently contaminated with sulfate to obscure a minimal need. Chemical test for sulfate in the deficient medium was negative, however. Nevertheless, methods to be reported (17) have shown a definite methionine requirement in this system.

Amino Acids.—All of the naturally occurring amino acids were tried, singly, as supplements to F. Those amino acids which consistently gave stimulation were *l*(+)-isoleucine, *l*(−)-phenylalanine, *l*(+)-aspartic acid, *l*(−)-proline, *l*(+)-lysine, *l*(+)-valine, *l*(+)-arginine, and *l*(+)-glutamic acid. Some of these results are presented in Figs. 4 and 5. At 25 mg. per cc. these compounds or their racemic mixtures caused a decrease in latent periods and slight and somewhat variable increases in burst size. The most marked stimulatory effect was noted with glutamic acid. The latent period in F supplemented with 25

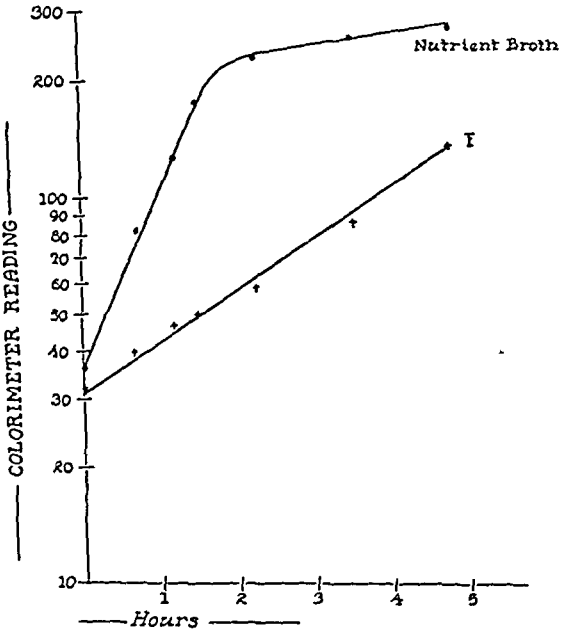

FIG. 2. Increase in turbidity of cultures of broth-grown bacteria in the broth and F media.

mg. per cc. of *l*(+)-glutamic acid was about 35 minutes, which in some cases was nearly 40 minutes shorter than the time required for infected cells in F medium alone. The burst size was frequently not increased but with a few cultures of bacteria was 4 or 5 times the control in F. Glutamine did not appear to be stimulatory.

l-tyrosine, *l*-histidine, and *d*(−)-valine, or *dl*-valine did not give reproducible stimulation. Certain amino acids were without effect. These included glycine, *l*(+)-alanine, *dl*-threonine, *dl*-methionine, and *l*(−)-tryptophane.

In contrast to these, *l*(−)-leucine and *l*(−)-serine inhibited virus synthesis. *l*(−)-cystine and *l*(−)-cysteine were toxic; *i.e.*, not only was virus not liberated but the number of infectious centers rapidly decreased.

The inhibition by *l*(−)-leucine was completely overcome by *dl*-valine, *dl*-

isoleucine, or *dl*-norleucine (Fig. 6). When one of these amino acids was added at 25 γ per cc. to infected bacteria in the presence of 25 γ per cc. of *l*(−)-leucine, even more virus was liberated and more rapidly than in infected bacteria in F.

TABLE I

The Effect of Omission of Single Constituents of F on Virus Multiplication in B_N-F

Time		
min.		
−90	0.10 cc. 24 hr. culture of B_N inoculated into 10 cc. N. Incubated at 37° with aeration. (B)	
	Removed six 2 cc. aliquots. Centrifuged 10 min. at 4000 R.P.M. Bacteria were suspended in: Tube 1 in 2 cc. F Tube 2 in 2 cc. F without Mg^{++} Tube 3 in 2 cc. F without $lactate^-$ Tube 4 in 2 cc. F without $SO_4^=$ Tube 5 in 2 cc. F without $PO_4^{\equiv}$ Tube 6 in 2 cc. F without NH_4^+	
	Centrifuged 10 min. Repeated twice. Assayed B	
0	0.90 cc. tubes 1–6 + 0.10 cc. T2r$^+$	Tubes a–f
5	0.10 cc. a–f + 9.9 cc. appropriate medium	Tubes 5a–5f
6	0.10 cc. 5a–5f + 9.9 cc. appropriate medium	Tubes I–VI
9	1.0 cc. I and III in centrifuge. Centrifuged 5 min.	
10	Assayed I–VI	
14	0.8 I–VI + 3.2 cc. appropriate medium	Tubes VII–XII
15	Assayed I–VI	
18	Assayed supernates I and III Assayed periodically on nutrient agar	

Inhibition by serine, cysteine, and cystine was not overcome by methylated compounds such as thymine or methionine.[1]

Glutamic Acid and the Antimetabolite, Methionine Sulfoxide

The marked stimulatory effect of glutamic acid suggested an essential rôle in the elaboration of virus. To demonstrate this, the effect of a specific glutamic

[1] We are indebted to Dr. Jack Schultz and Dr. Gerrit Toennies of the Lankenau Hospital Research Institute for some of the amino acids used in these experiments.

antimetabolite, *dl*, *dl*-methionine sulfoxide (i-MSO) was tested (18). For the following group of experiments, *E. coli* were grown in F medium. They were

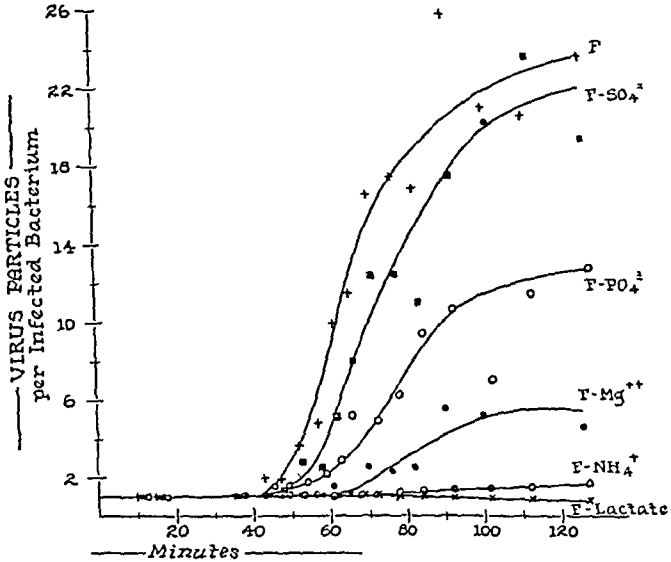

FIG. 3. The effect of omission of single constituents of F on virus production in F by broth-grown bacteria.

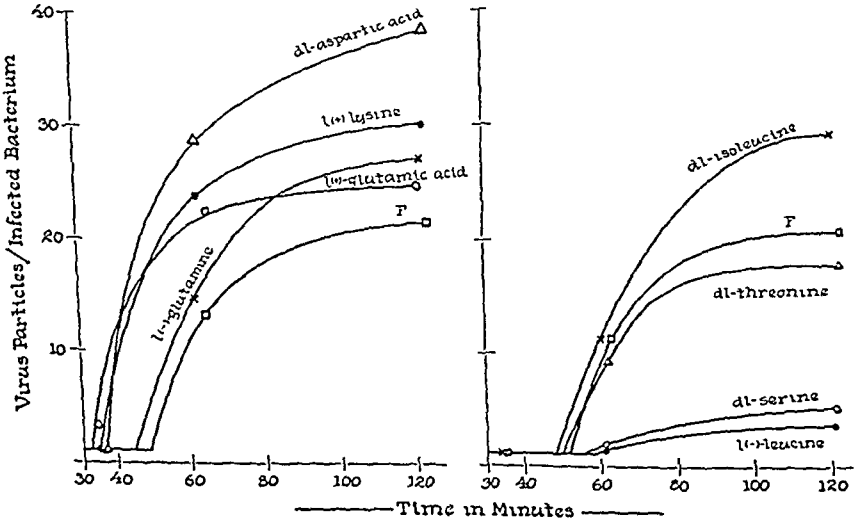

FIG. 4. The effect of some amino acids on the production of virus.

infected in the presence of i-MSO, or i-MSO was added at different times during the latent period of multiplication within the host. It was found that at high

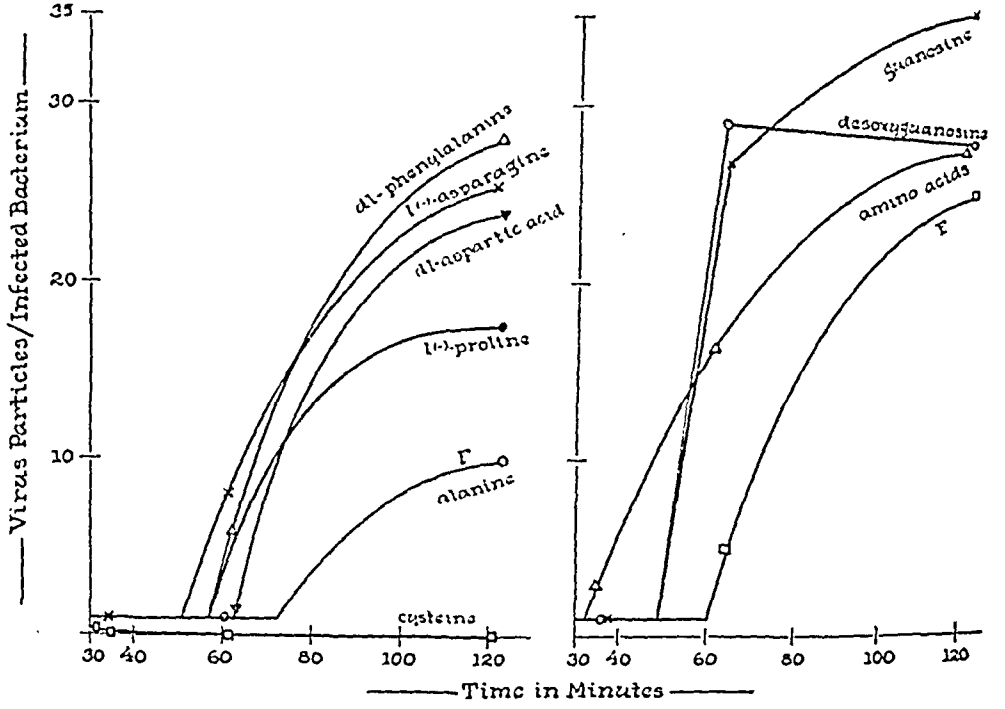

FIG. 5. The effect of some amino acids and nucleosides on virus production.

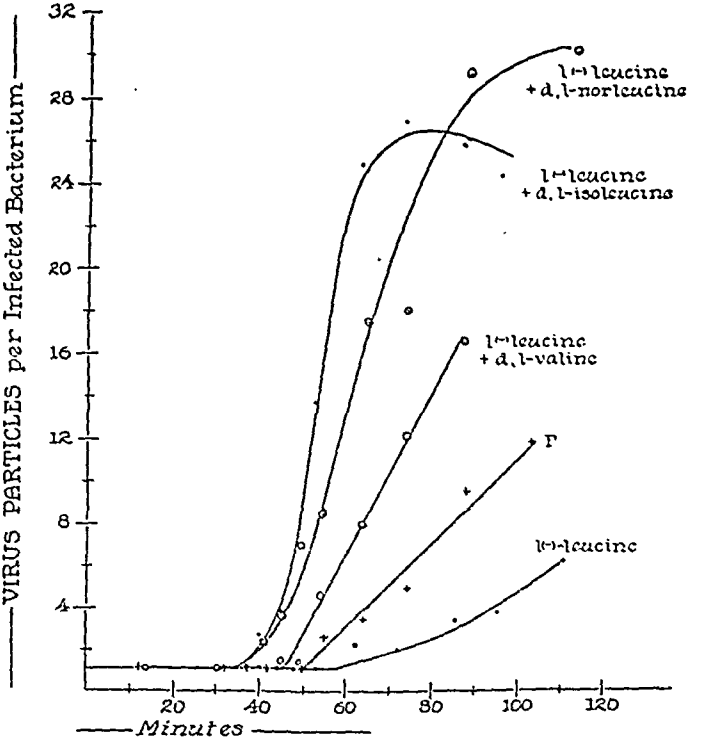

FIG. 6. The effect of some amino acids on the inhibition of virus production by leucine.

concentrations (5×10^{-2} M) i-MSO completely inhibited the liberation of T2 even if added after 17 minutes of the normal 25 minute latent period. At 5×10^{-3} M i-MSO, there was normal liberation of virus. The inhibition was reversed with glutamic acid or by diluting out the i-MSO in F. If bacteria were infected and allowed to incubate 6 minutes in the presence of i-MSO before ad-

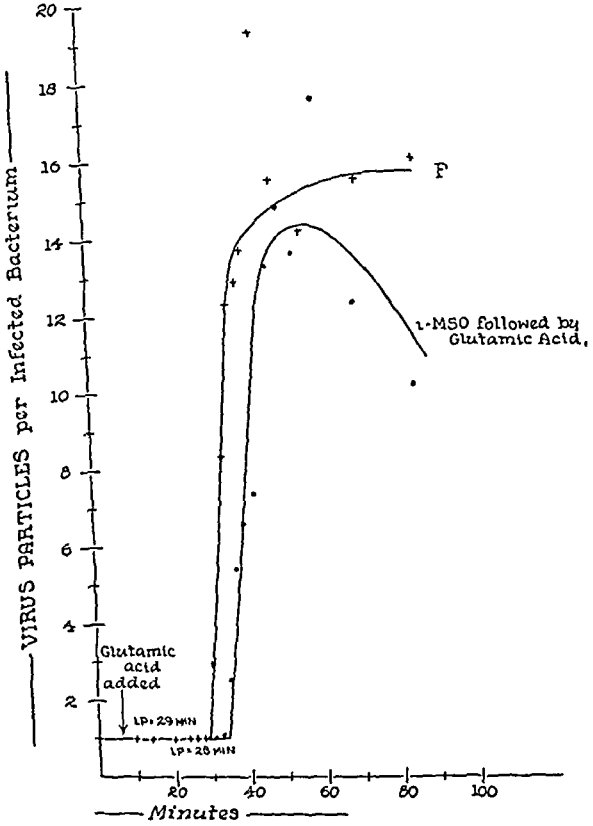

FIG. 7. The inhibition of virus production by i-MSO and the reversal of inhibition by the addition of glutamic acid.

dition of glutamic acid, it was found that virus was liberated 28 minutes after this reversal. The latent period of the control infected bacteria was 29 minutes (Fig. 7). There was a somewhat variable irreversible reduction in the number of infectious centers when the bacteria were left in i-MSO. These two effects, (1) the blocking of the start of virus synthesis, and (2) the "killing" of infected bacteria on continued exposure had previously been found to be characteristic of 5-methyl tryptophane. The effect of interrupting the latent period, as in studies with 5-methyl tryptophane (9), was further investigated. B_F were infected in F and after 10 minutes of the normal latent period, were inhibited

with i-MSO for 10 minutes. The i-MSO was diluted in F, and F containing 5×10^{-3} M glutamic acid. It was found that in either case virus was liberated a few minutes after the burst of the control bacteria, instead of the full 10 minutes required to complete the total normal latent period. The yield of virus from the interrupted bacteria about one-half the burst size of the normal bacteria. This may be explained by the "killing" of some of the infectious centers observed in this experiment during the 10 minute incubation with i-MSO. 5-methyl tryptophane, on the other hand, was shown to cause an absolute interruption of virus synthesis within the system, which on addition of tryptophane required the residual latent period and gave a normal burst size.[2]

Indole-3-Acetic Acid.—We have reported (19) that certain preparations of indole-3-acetic acid obtained from Eastman Kodak Company stimulated the production of T2 in B_N-F. Such stimulation was not found using samples from other sources (20) nor did possible contaminants such as indole, skatole, indolyl-1,3-diacetic acid, or Cu^{++}, which was used in the preparation of Eastman material (21), cause any stimulation. No other single substance, among more than 60 tested, produced as great a stimulation as we found for these particular preparations. 5×10^{-3} M indole-3-acetic acid itself, regardless of origin, inhibited virus multiplication.

Nucleic Acid Derivatives.—Fig. 5 shows the stimulatory effects of guanosine and desoxyguanosine. There were marked decreases in latent period and a somewhat variable increase in burst size in the presence of these compounds at 10 γ/cc. Depolymerized (*i.e.*, purified after alkaline extraction) preparations of ribose and desoxyribose nucleic acid showed similar effects on virus synthesis. The purine and pyrimidine bases, singly, at 10 γ/cc. were without marked effect.

Vitamins and Other Growth Factors.—Single vitamins were without significant stimulatory effect on B_N-F. Each vitamin was tested at only one concentration, with the exception of pyridoxine which was tested in the range of 0.005 to 5.0 γ per cc. Biotin—0.5 γ/cc., choline—5.0 γ/cc., cozymase—5.0 γ/cc., folic acid—0.5 γ/cc., glutathione—5.0 γ/cc., inositol—0.5 γ/cc., 2-methyl-1, 4-naphthoquinone—0.5 γ/cc., nicotinamide—2.0 γ/cc., para-aminobenzoic acid—0.2 γ/cc., Ca pantothenate—0.5 γ/cc., riboflavin—0.2 γ/cc., thiamine—3.0 γ/cc., sodium α-tocopherol phosphate—0.5 γ/cc. were used. It is possible that an optimal concentration which would cause stimulation was missed in this survey.

Inorganic Ions.—Cu^{++} and Fe^{+++} were toxic in concentrations as low as 10^{-5} M. The toxicity was overcome by dilution. At no concentration, however, was a stimulation of virus production found with these ions. Ca^{++} was also without effect in this system at concentrations below 10^{-3} M.

Complex Supplements.—On adding, at 500 γ per cc., a commercial casein hy-

[2] We wish to thank Dr. Heinrich Waelsch of the Department of Biochemistry of the New York State Psychiatric Institute, and Dr. Theodore Levine of the Lankenau Hospital Research Institute for samples of i-MSO and *l*-methionine *dl*-sulfoxide, respectively.

drolysate (Stearns "parenamine") fortified with 1.5 per cent tryptophane, a latent period of less than 30 minutes was always found, as well as an increase in burst size to nearly 50 per cent of that of B_N-N. Increasing the concentration

TABLE II

The Amino Acid, Purine, and Pyrimidine Composition of the Complete Defined Medium

Substance*	Concentration‡	Source
	γ/cc.	
Alanine	9.25	Bios
Arginine	18.6	Bios
Aspartic acid	30.0	Bios
Cystine	2.10	Bios
Glutamic acid	108	Merck
Glycine	2.25	Merck
Histidine	12.5	Pfanstiehl
Hydroxyproline	1.15	Bios
Isoleucine	48.3	Bios
Leucine§	58.0	—
Lysine	31.3	Merck
Methionine	16.3	Bios
Phenylalanine	19.0	Bios
Proline	43.3	Bios
Serine	25.0	Bios
Threonine	17.5	Bios
Tryptophane	5.00	Pfanstiehl
Tyrosine	26.6	Bios
Valine	39.7	Bios
Cytosine	10.0	Dougherty
Thymine	10.0	Schwartz
Adenine	10.0	Schwartz
Guanine	10.0	Schwartz

* All amino acids in this table were the natural isomers (*l* series).

‡ The concentrations of some amino acids were given by Stearns for parenamine. The concentrations of the other amino acids were taken from the values given for casein by Cohn and Edsall (22).

§ We wish to thank Dr. Joseph Fruton of the Department of Physiological Chemistry, Yale University, for the *l*(−)-leucine.

of parenamine did not cause further stimulation. It was found that parenamine was more effective than a mixture of naturally occurring amino acids at a total concentration of 500 γ per cc., and mixed in the proportions comparable to parenamine (Table II). There is considerable variation in the latent period and burst sizes in these two systems. Toward the end of these studies, subcultures of our original strain of *E. coli* B appeared to utilize the amino acid mixture more effectively than at the beginning. Parenamine, however, was always found to effect a speedier and greater synthesis of virus than the amino acid mixture alone (Fig. 8).

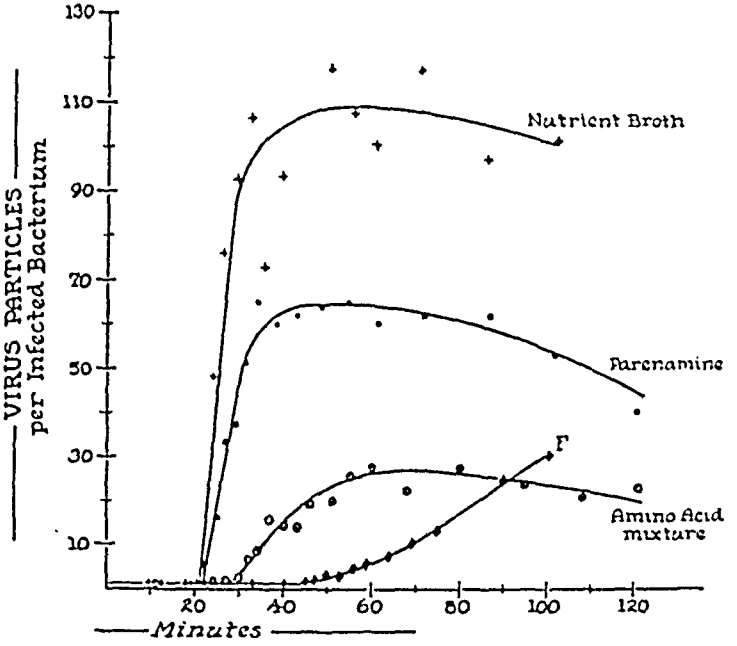

FIG. 8. A comparison of the stimulatory effects of parenamine and an amino acid mixture on virus production in F by broth-grown bacteria.

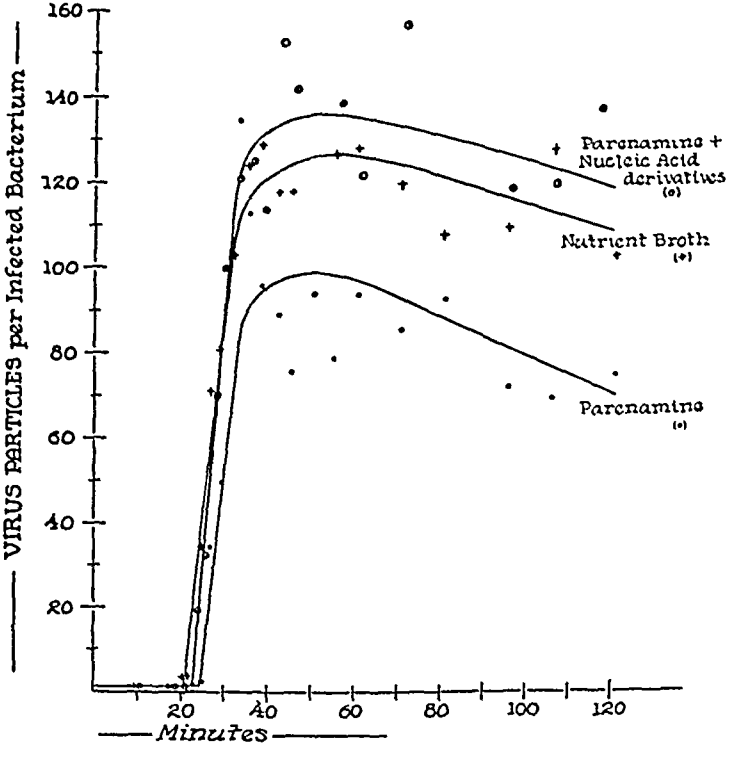

FIG. 9. The stimulatory effect of nucleic acid derivatives supplementing parenamine in the F medium.

The effect of adding a mixture containing adenine, guanine, thymine, and cytosine to parenamine is shown in Fig. 9. With such a mixture both burst size and latent period closely approximated those for B_N-N. When these purine and pyrimidine bases were added to a known amino acid mixture, there was also a marked stimulatory effect on latent period and burst size, as presented in Fig. 10.

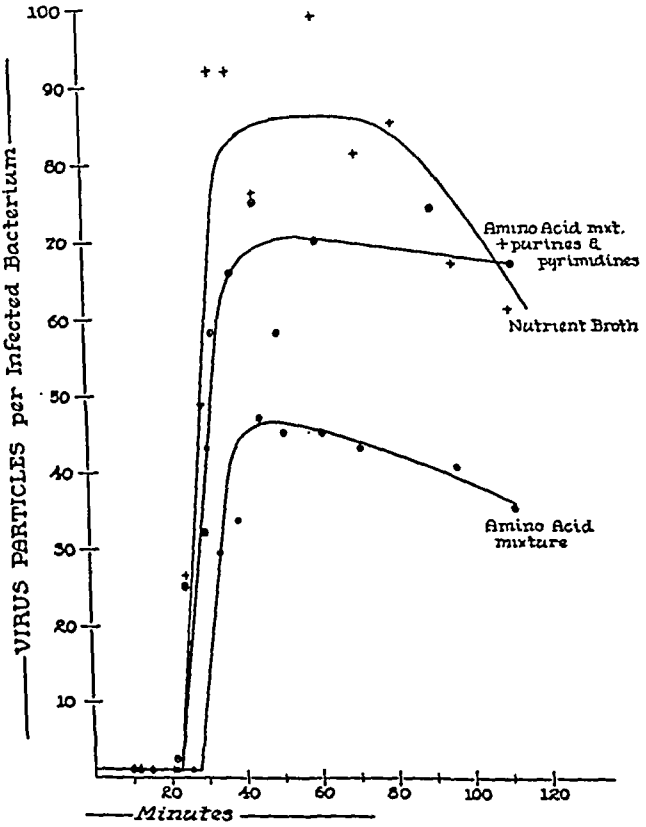

FIG. 10. The stimulatory effect of purines and pyrimidines supplementing an amino acid mixture in the F medium.

The addition of various vitamin mixtures to either of these complex supplements did not give reproducibly significant stimulatory effects.

It may be seen from Figs. 9 and 10 that the rate and amount of virus synthesized by B_N-N may be almost duplicated by a mixture of either parenamine fortified with purines and pyrimidines, or of the naturally occurring amino acids and purines and pyrimidines. With this mixture of chemically defined constituents, the latent period was within 3 minutes of, and sometimes the same as, the latent period in broth, and the burst size was rarely lower than 80 per cent of, and occasionally equalled, that of broth.

DISCUSSION

The time required to elaborate cellular components, such as proteins and nucleic acids, by *E. coli* B grown in nutrient broth (N) and transferred to the F medium was greatly increased. This is shown by the slower rate of increase in turbidity and the decreased rate of virus synthesis. It seems probable that those amino acids which, when added to B_N-F, caused a stimulation in the production of virus are the ones whose rate of synthesis in these bacteria under these conditions may be limiting factors in virus peptide synthesis. On the other hand, glutamic acid, which was the greatest stimulator of any of the amino acids, may be important in transamination, thereby facilitating the synthesis of other amino acids, or may be stimulatory because of the activity of derivative metabolites, such as α-ketoglutaric acid, as described by Spizizen (6). Thus, the stimulation by this amino acid when added as a single supplement to a simple medium may have resulted from an activity other than its function in the synthesis of virus in broth.

Other amino acids, also essential, would not necessarily be detected by this method. For example, tryptophane may be considered essential for virus synthesis since it was found in the virus, and 5-methyl tryptophane, a tryptophane antimetabolite, completely inhibited virus synthesis; however, tryptophane was not stimulatory when added as a single supplement to F. This merely signifies that other factors may have been more rate-limiting than tryptophane.

The method used has shown the nutritional requirements for synthesis of T2 to be multiple. External sources of both carbon (lactate) and nitrogen (NH_4^+) were necessary. The absence of phosphorus in the medium was severely limiting, confirming, in general, the results of studies with radioactive phosphorus which showed that most of the phosphorus incorporated in virus synthesized under somewhat different conditions was derived from the phosphorus-containing medium. The absence of magnesium also limited virus synthesis. On the other hand, the requirement for sulfur was not demonstrated by this method. Other methods, however, have shown a methionine requirement, which may serve as a sulfur source. In any case, although the omission of sulfur was found not to be limiting for virus synthesis by host cells in a limited medium, it might prove so for the greater synthesis of virus in the same host cell in a complete medium.

No single carbon- and nitrogen-containing compound was found which could effect a rate of synthesis of T2 in B_N-F characteristic of B_N-N. Thus the rate and amount of synthesis in B_N-N were approximated only by a complex mixture of *l*-amino acids, and purine and pyrimidine bases. Bacteria which have been grown in nutrient broth and washed, may still contain vitamins sufficient for the maximal utilization of known substrates. Under these conditions, supplementation with vitamins would not be expected to cause a stimulation. However,

a critical requirement for a vitamin in this system might be detected by the antimetabolite technique.

A survey of our data shows that the liberation of virus is to some extent independent of the amount of virus synthesized. Compounds which markedly decreased latent period did not always increase burst size.

The mechanism of the inhibition of virus synthesis by cystine, cysteine, leucine, and serine in this system is obscure. The inhibition by leucine was overcome by closely related amino acids. This same type of effect was observed by Gladstone (23) in the case of growth rate of *Bacillus anthracis* inhibited with serine and leucine.

SUMMARY

Using the one-step growth technique the production of the virus T2 in its host, measured by latent period and burst size, was shown to depend on the nutritional environment of the host cell.

When *E. coli*, grown in broth, was transferred to a simple medium, single organic compounds such as some amino acids and nucleosides were found to increase or accelerate the synthesis of virus.

An antimetabolite of glutamic acid, an amino acid important for virus synthesis, was shown to be inhibitory.

Several naturally occurring amino acids, leucine, serine, and cysteine, inhibited virus synthesis in the simple medium.

A chemically defined mixture was found which supported a rate of virus synthesis very nearly comparable to that found for host cells in nutrient broth.

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CHEMICAL STUDIES IN HOST-VIRUS INTERACTIONS*

V. Some Additional Methods of Determining Nutritional Requirements for Virus Multiplication

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(Received for publication, August 30, 1947)

It has been demonstrated in the previous paper (1) that the composition of the medium of the host cell affects the rate and amount of T2 bacteriophage synthesized in and liberated from infected *Escherichia coli*, strain B. The effects of numerous compounds on virus multiplication in a minimal medium (F) have been described; stimulatory effects by single supplementary compounds have been interpreted to signify that the compound plays a rôle in virus synthesis. Thus, a number of amino acids and nucleic acid derivatives were shown to be of interest as a result of this test. It was found that methionine sulfoxide, an antagonist of the stimulatory glutamic acid, interfered with virus reproduction.

A complex defined medium has been described consisting of the F medium supplemented with amino acids, purines, and pyrimidines, which almost duplicated the nutritive qualities of the broth medium for virus multiplication. The necessity for these amino acids, purines, or pyrimidines may be tested by studying the course of virus multiplication in the complex defined medium, in which single constituents are omitted. In this type of experiment, the course of virus multiplication has been followed by two methods: (1) the one-step growth technique previously described (1), and (2) the estimation of the synthesis of desoxyribose nucleic acid (DNA) in multiply infected cells in the appropriate media.

The use of DNA synthesis as a measure of virus multiplication in the T2 system follows from studies on synthesis in infected cells in F media. Thus it has been shown elsewhere (2, 3) that the onset and amount of DNA synthesis, which occurs at a constant rate, can be correlated with (1) the time of appearance of more than one virus particle within an infected cell, (2) the amount of virus liberated at 25 to 30 minutes, and (3) the amount of virus liberated by lysis-inhibited cells. The method has already been used to demonstrate the greater stimulatory value of supplementing the F medium with

* The work described in this paper has been aided by the Office of Naval Research.

a mixture of amino acids as compared to the most stimulatory single amino acid, glutamic acid (3).

Materials and Methods

Preparation of Bacteria.—*E. coli* B were grown in nutrient broth (N) as described previously (1). The bacteria, B_N, were grown to about 5×10^7 per cc. for one-step growth studies, and to 2×10^8 per cc. for studies of DNA synthesis in infected cells. The bacteria continued in a logarithmic phase with respect to division until about $1\text{-}2 \times 10^9$ per cc. However, the amount of protoplasm as followed turbidimetrically in a Klett-Summerson colorimeter with a 420 filter increased at the same logarithmic rate only until $2\text{-}4 \times 10^8$ per cc. and then increased at a much slower rate. Thus B_N studied in the one-step growth experiments were in their logarithmic phase with respect to division and growth, while B_N used in studies of DNA synthesis were approaching a change in synthesis affecting growth but not division. Nevertheless, B_N concentrations of 2×10^8 per cc. were chosen to avoid the use of large volumes of media, suspensions, and aliquots otherwise required for the DNA estimations.

The bacteria were washed twice in F medium and resuspended in the medium of desired composition at the concentration to which the bacteria had been grown. In almost all of the experiments, B_N from the same culture were used for the two different tests involving the same media.

Virus.—Purified concentrates of $T2r^+ - F$ and $T2r^+ - N$ in 0.85 per cent NaCl were used (4).

Media.—The amino acids used were all of the *l*-configuration. The complete defined medium has been described in the preceding paper (1). The relative proportions of the amino acids were arbitrarily chosen to approximate a casein hydrolysate in the minimal F medium (4). To this mixture were added adenine, guanine, cytosine, and thymine at 10 γ per cc. In the following experiments, virus synthesis in B_N was tested in the complete defined medium, the F medium, and the complete medium minus a single constituent

Analyses.—Virus production in singly infected cells in the various media was determined by the one-step growth technique of Delbrück and Luria (5). The course of DNA synthesis in the various media was determined by the application of the diphenylamine reaction to the trichloroacetic acid precipitates of infected cells (3).

RESULTS

One-Step Growth Experiments

In Table I is presented a summary of results obtained by this technique. It may be seen that there was very little change in the latent period on omission of a single constituent, despite the fact that with many amino acids the burst size or average number of virus particles per infected bacterium was significantly reduced. Although tests have not been carried out to determine whether all the infectious centers liberate virus on the depleted medium, it appears probable that they do. It is considered that a decreased burst size means a decreased number of particles synthesized per infected bacterium. The lack of correlation of latent period to burst size is striking; lysis appears to be almost independent of the number of virus particles in the cell.

In Fig. 1 are presented some experiments of this type. The effect of omission of leucine and tryptophane is readily apparent. Although tryptophane has been found in T2 (4), and its importance for multiplication affirmed by the

antimetabolite technique (6), tryptophane was not stimulatory in the single supplement study (1). It can be seen, however, that its omission from the complete medium markedly reduced burst size.

By the single supplement technique, leucine was found to be inhibitory although the inhibition was readily overcome by the action of isoleucine, norleucine, or valine (1). Despite its inhibitory action in one type of test,

TABLE I

Effect of Omission of Single Constituents

Compound omitted	Increase in latent period	Decrease in burst size	Approximate delay in DNA synthesis
	min.	*per cent*	*min.*
Alanine	0	0	0
Arginine	0	0	0
Aspartic acid	0	0	0
Cystine	0	0	0
Glutamic acid	0.5	38	3
Glycine	0	0	0
Histidine	1	45	6
Hydroxyproline	0	0	0
Isoleucine	2	47	0
Leucine	3	57	11
Lysine	0	0	0
Methionine	1.5	0	25–45
Phenylalanine	0.5	32	7
Proline	0	0	0
Serine	0	0	0
Threonine	0	0	0
Tryptophane	1	50	11
Tyrosine	0	0	7
Valine	1	66	6
Adenine	0.5	0	Variable
Cytosine	0	0	0
Guanine	0	0	0
Thymine	0	0	0

leucine was, nevertheless, important in virus synthesis, as detected by the omission technique.

Some compounds such as glycine or threonine did not show an effect when omitted from the complete medium. This need not mean necessarily these compounds are not incorporated into virus or are not involved in some intermediary rôle in virus synthesis. Since many compounds, such as desoxyribose phosphate, must be synthesized from lactate and inorganic phosphate to form virus, these results may merely signify that compounds such as glycine and threonine are synthesized at a faster rate than other rate-determining compounds, such as desoxyribose phosphate.

Desoxyribose Nucleic Acid (DNA) Synthesis

Eight amino acids have been found important by the one-step growth test described in the previous section: they include isoleucine, phenylalanine, tryptophane, leucine, valine, glutamic acid, methionine, and histidine. By following the course of DNA synthesis in infected cells, effects have been noted with all of these but isoleucine. In addition, omission of tyrosine and adenine produced effects on the course of DNA synthesis but not on the one-step growth curves.

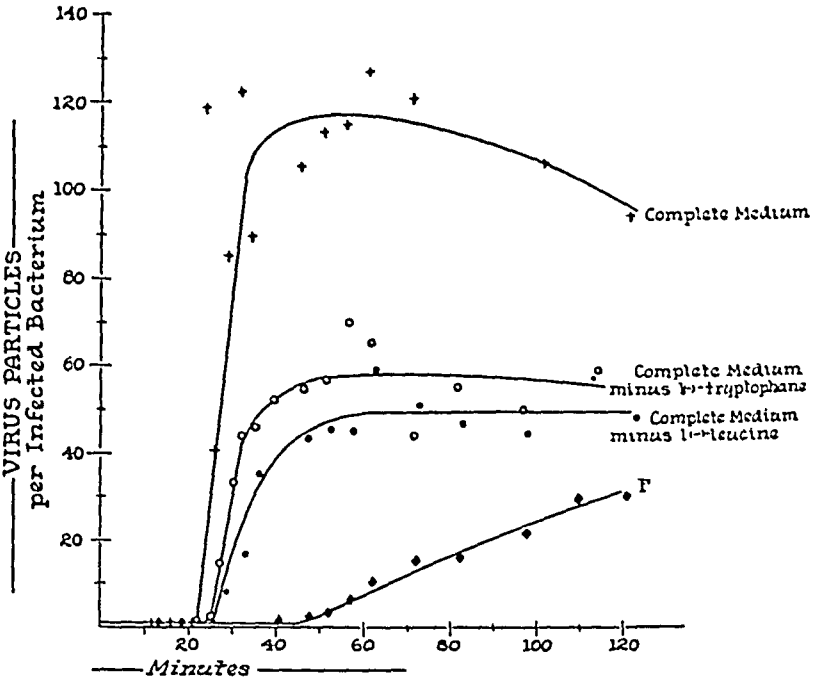

FIG. 1. The one-step growth curves of the T2r$^+$-*E. coli* system in media of different compositions.

In Fig. 2 are presented the curves for the synthesis of DNA in infected B_N suspended in the complete medium, the F medium, and the complete medium with tryptophane or leucine omitted. To B_N at 2×10^8 per cc. was added T2r$^+$ virus to give a final concentration of virus of 1×10^9 per cc. Two types of effect may be noted in this experiment. First, the omission of leucine or tryptophane resulted in significant delays in the onset of synthesis. Nevertheless, when synthesis began, it occurred at the same rate as in the complete medium. This type of effect has been noted consistently although the length of delay, as presented in Table I, depends on the amino acid. Secondly, the rate of synthesis fell off earlier in the case of the omitted amino acids. This was not noted frequently in 2 hour experiments. It was quite variable with the same amino acid and was only observed for tryptophane, leucine, and adenine.

Systems omitting adenine showed great variability. In several one-step growth experiments, no differences were noted from the complete medium. However, in four DNA synthesis experiments in media without adenine a marked delay of onset was observed in two, once the rate decreased sharply after 60 minutes, and in one experiment no difference was noted during the 2 hour interval.

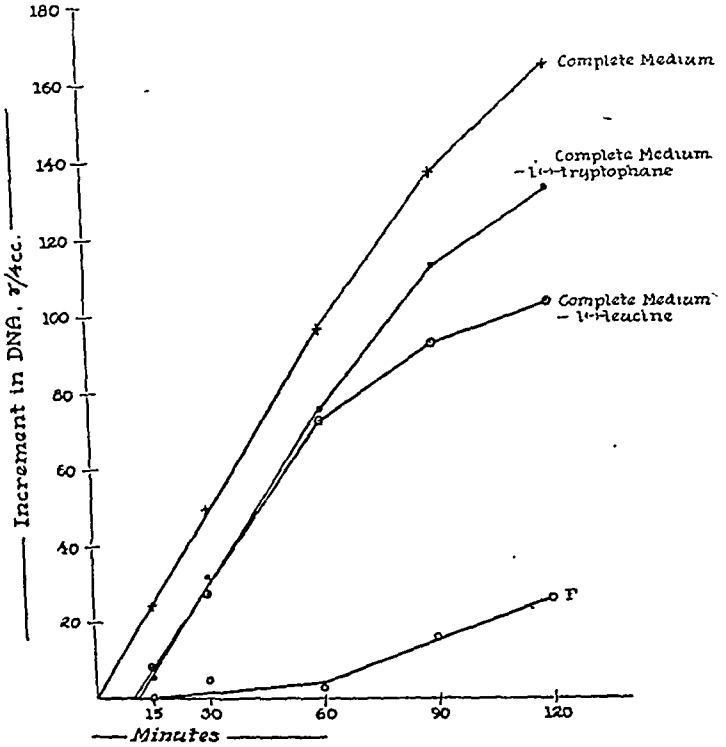

FIG. 2. The course of desoxyribose nucleic acid synthesis in the T2r$^+$-*E. coli* system in media of different compositions.

It was observed that the beginning of DNA synthesis in the complete medium varied from one culture of B_N to the next. This point is obtained by extrapolation of the linear increment of DNA to 0. In the experiment presented in Fig. 2, the curve was extrapolated very close to the origin. This occurred in a few experiments; in most cases, however, a latent period of up to 10 minutes was found.

In several experiments it was noted that the latent period of synthesis of DNA in F medium alone was exceedingly long; *i.e.*, about 80 to 90 minutes. The DNA formed after infection at this time in B_N at 2×10^8 per cc. could not

account for the virus liberated in comparable periods in this medium in the one-step growth test with B_N at 5×10^7 per cc. This effect is tentatively attributed to the decreased synthetic powers of B_N to the higher concentrations.

Under the conditions of this test, the effect of omission of methionine was the most marked of any of the amino acids, resulting in delays of synthesis of

TABLE II

Comparison of Results of Single Supplement and Single Omission Techniques

Compound	Effect of supplement	Effect of omission	
		One-step growth	Synthesis of DNA
Alanine	0	0	0
Arginine	+	0	0
Aspartic acid	+	0	0
Cystine	Inhibitory	0	0
Glutamic acid	+	+	+
Glycine	0	0	0
Histidine	Variable	+	+
Isoleucine	+	+	0
Leucine	Inhibitory	+	+
Lysine	+	0	0
Methionine	0	+	+
Phenylalanine	+	+	+
Proline	+	0	0
Serine	Inhibitory	0	0
Threonine	0	0	0
Tryptophane	0	+	+
Tyrosine	Variable	0	+
Valine	Variable	+	+
Adenine	0	0	+
Cytosine	0	0	0
Guanine	0	0	0
Thymine	0	0	0

The symbols + and 0 mean positive effect and no effect, respectively.

over 25 minutes. In contrast to this, one-step growth curves in media without methionine showed relatively small differences from the complete medium. This situation appears analogous to that described for F alone.

Simplification of the Complete Medium

As summarized in Table II, the single supplement technique indicated the need for seven amino acids, the omission techniques indicating a total of nine amino acids and one purine. Combination of these to include valine, isoleucine, leucine, phenylalanine, histidine, arginine, lysine, aspartic acid, glutamic acid,

methionine, tryptophane, tyrosine, and adenine as supplements to the F medium supported one-step growth curves comparable to that in the complete medium. The burst size was within 10 per cent of that in broth, the latent period being 1 to 2 minutes longer.

DISCUSSION

It is apparent that the techniques described in this paper permit the detection of compounds important for virus synthesis in addition to those revealed by the method previously described (1). By the three techniques as presented in Table II, thirteen amino acids and a purine have been shown to markedly affect the course of synthetic mechanisms tied to virus synthesis. However, the development of a medium for maximal virus synthesis in which the synthetic needs of the organism have been reduced to a minimum has not been completed. Compounds such as thymine or guanine are probably synthesized at a rate that precludes the detection of an effect on omitting them from the medium. The detection of a rôle for these compounds in virus synthesis might be accomplished by the use of competing structural analogues or more demanding host cells, such as mutant strains incapable of these syntheses.

In a host cell such as *E. coli* strain B capable of such varied synthesis, the interpretation of all the results is difficult. The more rigorously defined requirements of isolated animal cells, or of many bacteria may be expected to reveal many all-or-none effects in virus synthesis, which might be more easily interpreted.

In most of the experiments described in this paper the rate of DNA synthesis accounted very well for the amount of virus produced. For instance, the most active T2 preparation isolated in this laboratory contained 1.6×10^9 active particles per γ DNA. It may be calculated from the data in Fig. 2 that the amount of DNA synthesized at 30 minutes in the complete, tryptophaneless, and leucineless media accounted for burst sizes at that time of 99, 63, and 55, respectively. The observed burst sizes as presented in Fig. 1 were 118, 58, and 50, respectively. However, some conflicting results have been recorded. DNA synthesis did not correspond to virus production in the methionine-, tyrosine-, and isoleucine-deficient media. At present, these differences are attributed to differences in the age of the bacteria, and the difference in the ratio of virus to host cell in the two experimental systems.

SUMMARY

Omission of a single constituent from a chemically defined medium approximating the virus growth-promoting properties of broth affects virus production in infected bacteria. This may be estimated by the one-step growth technique and the course of desoxyribose nucleic acid synthesis. Nine amino acids and one purine have been shown to be important by these tests. A combination

of all constituents observed to be important by the single supplement and single omission techniques has approximated the virus growth-promoting properties of broth. Certain anomalous results have been commented upon.

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PRODUCTION OF PROTEINASE BY HEMOLYTIC STREPTOCOCCI IN VARIOUS CLINICAL CONDITIONS

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Elliott (1) described an extracellular proteolytic enzyme, streptococcal proteinase, in broth cultures of certain strains of group A streptococci grown under favorable cultural conditions. He observed that this enzyme digested the type-specific M protein antigen of group A streptococci as well as streptokinase, fibrin, milk, and casein. This enzyme was active under reducing conditions of bacterial growth; and in cell-free systems it was activated by cysteine, glutathione, potassium cyanide, and thioglycollic acid but was inactivated by iodoacetic acid and by normal mouse or rabbit serum. In a later report Elliott and Dole (2) found that the yield of active proteinase was greatest in broth containing undialyzed Pfanstiehl peptone and that cultures grown in media prepared with the dialysate of Pfanstiehl peptone or with undialyzed neopeptone produced the inactive precursor of streptococcal proteinase.

Todd (3) also demonstrated a streptococcal enzyme in culture filtrates of many strains of group A streptococci and found it to be antigenic. It was considered probably identical with the one described by Elliott.

It seemed important to determine the possible relationship of this newly discovered enzyme to the pathogenesis of streptococcal infections and their sequelae. A study was therefore undertaken to determine whether proteinase production by hemolytic streptococci isolated from patients with streptococcal infections as well as those isolated from normal carriers was in any way correlated with different types of clinical response. The capacity of various serological groups and types of hemolytic streptococci to elaborate proteinase was also investigated; and a study was made of the presence of proteinase as related to the presence of streptokinase in broth cultures of group A streptococci.

Materials and Methods

Source of Streptococcal Strains.—The majority of the strains were isolated at the Hospital of The Rockefeller Institute for Medical Research in 1940–47 from pharyngeal cultures of rheumatic and non-rheumatic subjects suffering from pharyngitis, scarlet fever, or rheumatic fever, or from throat cultures of normal carriers. Many of the patients developed purulent

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complications such as sinusitis, otitis media, peritonsillar abscess, cervical lymphadenitis, pneumonia, or impetigo; others developed rheumatic fever following their streptococcal infections. In a few instances both purulent complications and rheumatic fever were observed in the same patients. The carrier strains were cultured from rheumatic subjects and their siblings who developed no clinical evidence of disease and no streptococcal antibody response.

Other strains were isolated in the Chicago area at the Great Lakes Naval Training Station during the spring of 1946 from pharyngeal cultures of patients with streptococcal respiratory diseases including scarlet fever, some of whom later developed rheumatic fever. All these strains were frozen and dried shortly after isolation from the patients and kept in this state until used. A few lyophilized streptococcal strains were selected from laboratory stock cultures to complete the series of all the known serological groups and types; these strains had probably in many instances been subcultured numerous times before lyophilization.

Grouping and Typing of Streptococcal Strains.—The hemolytic streptococci were classified into serological groups and types by the precipitin technique (4, 5). In a few instances, strains which could not be classified by the precipitin method with the available sera were tested by cross-agglutination with homologous and heterologous rabbit antisera to determine whether they were antigenically similar or dissimilar.

Preparation of Cultures.—Each strain was seeded from the lyophilized stock of cultures into 5 cc. of Todd-Hewitt broth containing 2 per cent Pfanstiehl peptone or 1 per cent neopeptone and incubated in a water bath at 37°C. for 12 hours. A loopful of the actively growing culture was then transferred to 5 cc. of fresh broth and incubated for 28 hours at 37°C.

Determination of Proteinase Activity.—Pfanstiehl peptone broth was used exclusively to prepare cultures for determinations of proteinase activity unless otherwise stated. A method, described by Elliott and Dole (2), which employs the coagulation of a milk-thioglycollate mixture was used in most experiments. Preliminary studies revealed that whole broth cultures gave results for proteinase assay comparable to those obtained with filtrates or supernates of these cultures. Because of simplicity, whole broth cultures were therefore employed. Control cultures were included in each series of tests, namely, a positive strain (K43) and a negative strain (K43 mouse passage).

In a few experiments the "azocoll" digestion technique (3) was carried out in parallel with the milk-thioglycollate method to estimate proteinase activity of streptococcal cultures.

Estimation of Streptokinase.—The method employed for the estimation of streptokinase was a modification of that described by Tillett, Edwards, and Garner (6) and the Commission on Acute Respiratory Diseases (7).

The cultures of streptococci prepared for proteinase determination were tested for streptokinase by mixing gently 1.0 cc. of filtrate with 1.0 cc. of 0.6 per cent solution of a lyophilized fibrinogen, fraction I (Cohn)[1], from human plasma in physiological saline, buffered with veronal at pH 7.5 and 0.2 cc. of bovine thrombin in a 1:10 dilution of the same buffer. The mixtures were incubated in a water bath at 37°C. The streptokinase activity of the various strains was determined by liquefaction of the standard fibrin clot. Liquefaction was considered to have taken place when the clot moved or flowed freely on inversion of the tube.

OBSERVATIONS AND RESULTS

Proteinase Capacity of Group A Streptococci in Relation to the Clinical Response.—The proteinase-producing capacity of strains of group A streptococci isolated from patients with various clinical responses to streptococcal infection and from the throats of normal carriers is summarized in Table I. Of 155

[1] Fibrinogen human fraction I was obtained through the courtesy of Sharp and Dohme.

strains cultured from 144 patients or carriers between October, 1940, and April, 1947, 70 produced proteinase and 85 did not produce any of this enzyme. At different times 8 patients suffered more than one streptococcal infection. These were considered separate infections because the same serological type was not observed in the recurrent infections, and because significant rises in the antistreptolysin O titre of the patients' sera occurred. In the case of carriers, observations were confined to a single strain from each individual unless a change in serological type was observed. All streptococcal strains were isolated from nasopharyngeal cultures within 5 days after the onset of infection with the exception of those from 9 patients with a primary attck of rheumatic fever from whom cultures were not obtained until 2 to 4 weeks after infection. Seventy-three of the strains included in Table I were isolated from patients with scarlet fever. These are not grouped separately because the only difference between the patients with scarlet fever and those with pharyngitis alone was the erythematous rash.

Of 47 group A streptococcal strains cultured from patients who made uneventful recoveries from their acute pharyngitis, 25 strains produced proteinase and 22 failed to elaborate this enzyme. From patients with purulent complications, 15 group A streptococcal strains were isolated and all except one strain failed to produce proteinase. Ten of the 14 strains which did not elaborate the enzyme, however, were all serological type 19 and were isolated from the same epidemic. Apparently these strains were derived from a common source. Among 34 strains isolated from patients who developed a primary attack of acute rheumatic fever, 15 produced proteinase and 19 did not. In this group, 3 strains were cultured from patients who developed both purulent complications and rheumatic fever.

In Table I are also recorded the results of the proteinase capacity of a series of strains isolated from the nasopharynges of rheumatic subjects who suffered hemolytic streptococcal infections of the upper respiractory tract. The strains from those patients who developed a recurrence of rheumatic fever can be contrasted with strains isolated from patients who did not develop rheumatic fever following the streptococcal infection. Of 19 strains, each isolated from a different infection followed by an attack of rheumatic fever, 9 produced proteinase and 10 did not. Among the patients who did not develop rheumatic fever, there were 23 infections. Ten strains from these infections produced proteinase and 13 failed to show any activity.

One instance is of interest because the same strain, producing proteinase *in vitro*, induced different clinical reactions in susceptible rheumatic subjects. A group A streptococcus of an unclassified type was cultured from a rheumatic subject with an attack of acute pharyngitis that was not followed by rheumatic fever; this same type caused an accidental respiratory infection in another rheumatic susceptible subject, who subsequently developed rheumatic fever.

The streptococci from these patients were shown to be the same type by cross-agglutination tests with homologous and heterologous rabbit antisera.

The proteinase capacity of streptococcal strains cultured from the throats of subjects who developed no apparent infection is also shown in Table I. These individuals were observed at 3 to 4 week intervals for several years, and no clinical or laboratory evidence of infection appeared when these strains were isolated. Moreover, no elevated titres for antistreptolysin O, antifibrinolysin and, in a few cases studied, bacteriostatic antibodies were observed in the sera

TABLE I

Proteinase Production by Group A Streptococci as Related to Clinical Response to Streptococcal Infection

Clinical response to streptococcal infection		Proteinase production by streptococci isolated from individual patients	
		No. of strains	
		Positive	Negative
Pharyngitis:	Uncomplicated	25	22
"	With purulent complications	1	14*
"	Followed by primary attack of rheumatic fever	15	19
"	Followed by recurrence of rheumatic fever in rheumatic subjects	9	10
"	Followed by no recurrence of rheumatic fever in rheumatic subjects	10	13
No infection:	Normal carriers	10	7
Total		70	85

* Ten of these strains were isolated from the same epidemic and were of the same serological type.

of these subjects. Of 17 strains from these individuals, 10 strains had demonstrable proteinase activity but 7 were proteinase-negative.

Upon consideration of the data presented in this table, there appears to be no evidence that proteinase- or non-proteinase–producing strains were associated with any particular variety of streptococcal infectious process: Strains with or without proteinase activity were each isolated from normal carriers or from patients who developed rheumatic fever, purulent processes, or uncomplicated infections.

Proteinase Production of Epidemic, Endemic, and Carrier Strains of Group A Streptococci.—An opportunity presented itself to investigate the proteinase capacity of epidemic, endemic, and carrier strains of various serological types of group A streptococci. The epidemic strains were isolated from young adult

Naval personnel in New York City from 1942–44 and in the Chicago area during 1946. The endemic and carrier strains were obtained during 1940–47 from children and young adults in New York City who were in no contact with those from whom the epidemic strains were cultured. In Table II is shown the proteinase production of these 3 groups of strains. A variety of serological types are represented in each group and the time of isolation is spread over relatively similar dates. Twelve of the endemic and 11 of the carrier strains could not be typed by the precipitin method with the available sera, and are therefore not included in the number of serological types but are included in the number of strains investigated.

It is apparent from the results shown in Table II that irrespective of whether the streptococci are of epidemic, endemic, or carrier source, there is approxi-

TABLE II

Proteinase Production of Group A Streptococci as Related to Their Sources: Epidemic, Endemic, and Carrier Strains

Source of streptococci	No. of strains	No. of serological types	Proteinase production by individual strains	
			Positive	Negative
Epidemic	91	16	37	54
Endemic	47*	18	24	23
Carrier	17*	5	10	7

* Twelve of the endemic and 11 of the carrier strains could not be typed with available sera; the number of types represented by the remainder is indicated in the third column.

mately an equal distribution of proteinase- and non-proteinase-producing strains. Moreover, it can be seen that 16 different serological types are included in the epidemic group. If, on the other hand, a single serological type from an epidemic and, presumably, from a common source is assayed, a uniform pattern of proteinase production will be observed. Examples of this will be presented in Table III.

Lack of Correlation between Proteinase Capacity of Serological Types of Group A Streptococci and Clinical Manifestations.—To ascertain whether there is any correlation between the proteinase activity of strains belonging to a single type and the clinical reaction to infections induced by these strains, numerous serological types of streptococci were studied. In Table III are presented 5 representative examples to illustrate the proteinase production of strains of the same serological type cultured from patients whose clinical response to streptococcal infections varied. It can be seen that the serological types 1, 3, and 19 comprise both proteinase- and non-proteinase-producing strains; and in these types, strains of the same serological type and proteinase activity frequently induced different clinical manifestations. The same thing was observed with

the type 30 strains all of which, isolated from a single epidemic, produced proteolytic enzyme. In contrast to this, most of the type 17 strains, chiefly from the same epidemic, did not produce proteinase but were, nevertheless, able to initiate a variety of clinical effects.

On the other hand, as exemplified in type 19 (Table III), strains of the same serological type but differing in their ability to produce proteinase, were often found to give rise to the same clinical manifestations in different patients. Similar results were obtained with 17 other serological types not recorded in

TABLE III

Lack of Correlation between Proteinase Production by Group A Streptococci of Various Serological Types and Clinical Response to Infection

Clinical response to streptococcal infection	Proteinase production by individual strains of streptococci of various serological types isolated from patients with different clinical responses to infection									
	Type 1		Type 3		Type 17		Type 19		Type 30	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Pharyngitis: Uncomplicated			3			6	2	12	8	
" With purulent complications								10	1	
" Followed by primary attack of rheumatic fever	1		1			3	5	12	4	
" Followed by recurrence of rheumatic fever in rheumatic subjects	1					2		2		
" Followed by no recurrence of rheumatic fever in rheumatic subjects		1		2	1			1		
No infection: Normal carriers		2		1						
Totals	2	3	4	3	1	11	7	37	13	0

Table III. It is noteworthy that serological types 14, 19, and 26 included strains which produced no proteinase and were each associated with either the presence or absence of rheumatic fever recurrences in susceptible subjects.

The majority of the strains belonging to the serological types 17, 19, and 30 which showed uniformly positive or negative proteinase activity were isolated during epidemics of streptococcal infections and are probably derived from common sources. The fact that these epidemic strains of the same serological type each had their origin from a single parent strain and induced a variety of clinical reactions to infection emphasizes the lack of correlation between the proteinase capacity of the serological type and the disease state.

Comparison of Proteinase Capacity of Strains Isolated in Acute and Convalescent Phases of Infection.—A series of strains isolated from patients at weekly

intervals during the acute phase of illness through convalescence was selected to determine whether any change in proteinase capacity occurred. In the test for this activity the time at which coagulation of the milk first occurred gave a roughly quantitative estimate of proteinase production. Eight serial weekly cultures from a patient with a type 38 infection and 12 from a patient with a type 26 infection showed a uniformly negative response when tested for proteinase production; 5 weekly cultures from a patient with a type 12 infection, 8 from a patient infected with an unclassified strain, and 6 cultures from a patient with a type 30 infection showed positive reactions which were quantitatively consistent. In addition, from 4 to 7 weekly cultures of 3 sulfadiazine-resistant strains from patients with type 17 infections were tested for consistency of enzyme production by cultures isolated in the acute phase through convalescence. None of these cultures produced proteinase.

The majority of the patients from whom epidemic strains were obtained at the Hospital of the Rockefeller Institute received therapeutic doses of sulfadiazine for 7 to 14 days in an effort to clear up the carrier state or purulent complications. Cultures were taken from patients both before and after sulfadiazine therapy to observe any possible change in enzyme production due to the action of the drug. Proteinase-positive cultures were isolated both before and after therapy from 4 patients, two with type 23 infections, and one each with a type 30 and a type 3 infection. Proteinase-negative strains were similarly isolated from 6 patients, 3 with type 19 infections and 3 with infections of types 6, 14, and 38 respectively. In all cases the proteinase activity remained unchanged. The remarkable uniformity of proteinase production of these strains while they remained in the nasopharynx of patients suggests that the capacity to produce this enzyme is reasonably stable.

Proteinase Production by Various Serological Groups and Types of Hemolytic Streptococci.—To determine the relationship between the serological groups and types of hemolytic streptococci and proteinase production, a series of strains were tested from multiple sources which included the strains of group A streptococci previously considered in this report. In addition, other strains selected from laboratory stock cultures kept in the lyophilized state for many years were included.

A total of 238 strains, of which 208 belonged to group A and 30 to other established Lancefield groups,[2] was assayed. There were 41 known serological types of group A streptococci represented; and 23 group A strains were not classified into types with the available sera. The capacity to clot milk appeared to be limited to group A strains with the exception of one group D strain, D76, probably bovine in origin, and one group M strain, D168A"X", isolated from the vagina of a normal dog. Both of these strains were tested in

[2] Groups B, C, D, E, F, G, H, K, L, M.

duplicate and on different days; in each test, milk was clotted between 4 and 24 hours. The pH of the cultures was 6.8, which excludes acid clotting of the milk. The possibility that some other enzyme system might also cause clotting of milk must be considered. Certain group D strains, notably *S. liquefaciens*, are known to have proteolytic properties which may be different from the one studied in group A streptococci.

Of 208 group A strains tested, 107 or 51 per cent produced the enzyme. Since 23 strains could not be typed, a total of 185 strains, distributed among 41 different serological types of group A streptococci, is tabulated in Table IV. Of the types represented, 35 had more than one strain and of these, 11 types (1, 2, 3, 4, 17, 19, 24, 28, 29, 43, and 46) comprised both proteinase-positive and negative strains. The large number of positive- or negative-enzyme-producing strains in types 17, 19, and 30 is due to the fact that they are epidemic strains and probably originated from a common strain of the corresponding type. From these data it appears that many of the serological types of group A streptococci include strains capable of producing proteinase and others which do not produce this enzyme. However, further studies are necessary before such variations can be established as characteristic of the type.

Relationship between the Presence of Proteinase and Streptokinase in Broth Cultures.—Since Elliott (1) has demonstrated that streptococcal proteinase digests streptokinase, the relationship of these enzymes in cultures was investigated. In Table V is shown the reciprocal relationship between the presence of proteinase and the demonstration of streptokinase in cultures of various strains of hemolytic streptococci grown in Pfanstiehl peptone broth. The proteinase production was determined by both the milk-coagulation and azocoll tests; and the results of the measurement by these two methods were identical. The pH of the filtrates varied from 5.9 to 6.3. The reaction was never low enough to cause acid clotting of milk. A total of 37 strains of 9 different serological types of group A streptococci was tested in Pfanstiehl broth. Of these, 20 strains produced proteinase and no streptokinase, while 17 strains were proteinase-negative and streptokinase-positive.

In the present study advantage was taken of the observations of Elliott and Dole (2) to determine whether the reciprocal relationship between the presence of proteinase and streptokinase would be maintained in both Pfanstiehl and neopeptone broth. Ten strains of 5 different serological types were tested in both broths. In 7 of these strains, proteinase but no streptokinase was found in Pfanstiehl broth, and streptokinase but no proteinase was detected in neopeptone broth. The results in the other 3 strains were variable. It appears that in strains which produce both proteinase and streptokinase the reciprocal relationship between these two streptococcal components is, in most cases, dependent on the conditions in the culture media suitable for the production of the inactive or active proteinase: Thus in neopeptone broth the streptococcal

TABLE IV

Proteinase Production by Group A Streptococci of Various Serological Types

Serological type	No. of strains	Proteinase production	
		Positive	Negative
1	5	2	3
2	2	1	1
3	8	5	3
4	3	1	2
5	3	3	0
6	7	0	7
8	3	3	0
9	2	2	0
11	2	2	0
12	3	3	0
13	3	3	0
14	5	0	5
15	3	3	0
17	16*	1	15
18	3	0	3
19	44*	7	37
22	3	3	0
23	3	3	0
24	6	5	1
25	1	1	0
26	5	0	5
27	1	1	0
28	3	2	1
29	3	1	2
30	13*	13	0
31	2	2	0
32	3	0	3
33	3	3	0
34	1	0	1
35	2	2	0
36	3	3	0
37	1	0	1
38	3	0	3
39	1	1	0
40	3	3	0
41	1	1	0
42	2	2	0
43	3	2	1
44	3	3	0
46	3	1	2
47	1	1	0
Totals	185	89	96

* The majority of these cultures were epidemic strains and in each type were probably derived from one or two sources.

cells produce the proteinase as a precursor which is inactive and streptokinase is not digested; whereas in Pfanstiehl broth, the active proteinase is formed and the streptokinase is digested.

DISCUSSION

A unique opportunity was provided for obtaining epidemic strains of several serological types of group A streptococci from patients with a variety of clinical responses to infection. These strains were isolated from young adults in the second decade of life who lived under similar conditions while in Naval

TABLE V

Relationship between the Presence of Proteinase and the Demonstration of Streptokinase in Cultures of Group A Streptococci

Serological type	No. of strains	Proteinase*	Streptokinase
1	1	+	−
3	2	+	−
6	1	−	+
17	7	−	+
19	5	+	−
19	9	−	+
24	1	+	−
30	9	+	−
33	1	+	−
36	1	+	−
Total strains.....	37	Proteinase-positive.....20 Proteinase-negative.....17	Streptokinase-positive.....17 Streptokinase-negative.....20

Symbols + or − indicate the presence or absence of proteinase or streptokinase.

* The results of the measurement of proteinase production by milk-coagulation and azocoll titration were identical.

service. Moreover, a relatively large number of these epidemic strains were of a few serological types, in each of which the capacity of the streptococci to produce proteinase was uniform. The epidemic conditions suggested common origins for the strains within each type and made possible a study of the effect of proteinase production upon the clinical manifestations in patients, uncomplicated by other differences in the streptococci.

The data obtained revealed no evidence that proteinase or non-proteinase-producing epidemic strains of various serological types were associated with any particular variety of infectious process. This was indicated by the fact that strains of the same serological type and proteinase activity were isolated from patients who developed various clinical manifestations. On the other hand, strains which differed only in their ability to produce proteinase showed no difference in the pathologic conditions evoked in individual patients.

An especially suitable series of streptococcal strains from endemic sources was available for comparison with the epidemic strains described above. The endemic and carrier strains were derived from individuals who had had previous attacks of rheumatic fever and from their siblings. All of this group were followed constantly for several years as previously reported (8). Since a study of these strains showed the same findings as those from epidemic sources, it appears to be a general phenomenon that there is no correlation between the capacity of group A streptococci to produce proteinase and the clinical reactions of the patients to infection with these strains.

The possibility was considered that strains long resident in the throats of patients convalescing from infection might change in their function to elaborate this enzyme. Rather than any change being observed, a remarkable stability of these strains to produce a quantitatively uniform amount of enzyme over long periods was demonstrated. This stability was apparent even in the presence of therapeutic doses of sulfadiazine administered to the patient.

The capacity to produce proteinase appears to be related to the strain rather than to the serological type, as indicated by the occurrence of both enzyme- and non-enzyme-producing strains in many of the serological types. In some of the types investigated, all of the strains were similar in their ability to produce enzyme. The fact that in each instance there was intermingling of Naval Personnel between stations in which epidemics occurred suggests that these strains were probably derived from a common parent strain in each type.

To determine whether proteinase production is characteristic of individual types, it will be necessary to test many more strains from unrelated sources. In considering this relationship, it is of interest that Todd (3) found proteinase activity in concentrated filtrates which showed no activity before concentration. This suggested that most strains may produce the enzyme under suitable conditions.

The reciprocal relationship between the presence of proteinase and that of streptokinase in streptococcal cultures revealed a situation analogous to that observed by Elliott (1) with the type-specific M antigen of group A streptococci. Proteinase digests both streptokinase and the M protein; therefore, the two latter streptococcal components are not usually found in the same cultures with proteinase. Nevertheless, it has been observed that occasionally streptokinase may be present in small amounts simultaneously with proteinase, a situation also found to hold true with regard to the balance between the presence of M protein and proteinase in the same system (1). In the case of the two enzymes, one of which destroys the other, presumably the streptococcal cell may maintain constantly its function of producing both, but the balance between their relative concentrations is determined by the conditions prevailing in the culture at the time of testing.

SUMMARY

The proteinase activity of strains of group A streptococci isolated from patients in acute and convalescent phases of illness was found to be remarkably constant. Sulfadiazine therapy of patients did not affect proteinase production by the infecting streptococci.

The ability to produce streptococcal proteinase would appear to be related to the strain and not necessarily to the serological type.

No relationship could be found between the capacity of group A streptococci of different serological types to produce proteinase and the various clinical responses to infection with these microorganisms.

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IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS*

VI. The Cross-Reaction between Type XIV Antipneumococcal Horse Serum and Purified Blood Group A, B, and O Substances from Hog and Human Sources

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The agglutination of human erythrocytes of blood groups A, B, and O by Type XIV antipneumococcal horse sera has been clearly demonstrated (1, 2). Purified blood group A substance from commercial pepsin precipitates with Type XIV horse antiserum (3) and in one instance, removed about one-half of the antibody from an antiserum. In addition, both the specific polysaccharide of the Type XIV pneumococcus (SXIV) and, after partial hydrolysis, the blood group A substance react with horse antianthrax sera (4). The chemical basis for these cross-reactions has been clarified by the finding that the blood group A substance, SXIV, and the C polysaccharide of the anthrax bacillus contain in common N-acetyl glucosamine and galactose (3, 4). With the demonstration that purified blood group A and O substances from individual hog stomach linings were identical in a considerable number of chemical and physical properties (5) and that both contained *N*-acetyl *d*-glucosamine, *d*-galactose, and *l*-fucose (6) it was considered that a quantitative study of the precipitin reaction of these substances with Type XIV antipneumococcal horse serum might provide information about their structural similarities and differences. The surprising result was that preparations of hog blood group A substance differed widely in their capacity to precipitate anti-SXIV although they were all of equal purity and had the same capacity to precipitate homologous anti-A formed in man (5). Similar variations in precipitability for anti-SXIV were found among various preparations of hog blood group O substance, and of blood group A and O substances from human saliva and stomach (7).

EXPERIMENTAL

The blood group A and O substances from individual hog stomach linings and the blood group A substances from human saliva, stomach, and amniotic fluid were the preparations

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that had been used in previous studies (5–7). In addition, samples of blood group substances were purified from human B and O saliva and another preparation of O substance was obtained from a human stomach by the method described for the isolation of the blood group A substance (7).

Two Type XIV antipneumococcal horse sera were used; A66, supplied by Dr. A. J. Weil of Lederle Laboratories contained both Type XIV and Type XIX antibody, and H635 (1939 bleeding) provided by the New York State Department of Health Laboratories. The two sera contained 1.08 and 0.87 mg. anti-SXIV N per ml. and both sera agglutinated human erythrocytes of blood groups A, B, and O.

The cross-reaction between the various blood group substances was studied by adding increasing quantities of blood group substance dissolved in saline to a series of 10 ml. conical centrifuge tubes each containing a measured volume of antiserum at 0°C.; the total volume was kept constant. The contents of the tubes were mixed and the tubes kept in the refrigerator for a week (8) during which period their contents were mixed twice daily. The precipitates were then centrifuged off in a refrigerated centrifuge, washed twice in the cold with chilled saline, quantitatively transferred to 10 ml. micro-Kjeldahl flasks with water and a few drops of M/2 NaOH, and analyzed for nitrogen by the Markham micro-Kjeldahl method (9). Values represent the average of duplicate analyses. Supernatants from each pair of analyses were combined and divided in half. To one portion 25 or 50 μg. blood group substance was added to test for residual cross-reacting antibody and to the other portion 0.1 ml. of antiserum was added to test for excess blood group substance. Supernatant tests were set up at 0°C., left in the refrigerator for 1 week, centrifuged in the cold, and the degree of precipitation in each tube was noted.

RESULTS

From Table I it is evident that individual preparations of hog blood group A substance vary greatly in their capacity to precipitate with Type XIV antipneumococcal horse sera. For instance, 500 μg. of the blood group A substance from hog 10 precipitated only 19 μg. N from 0.5 ml. serum H635 while equal amounts of similar products from hogs 16, 3, and 8 precipitated 39, 72, and 72 μg. N respectively. The same range of variation in precipitating power was also observed with hog blood group O substances. Tests on supernatants were carried out in all instances and invariably showed a broad zone of precipitation indicating the presence of both antigen and antibody. Several representative sets of supernatant tests are shown in Table I. From the data with hog 16 and hog 29, it is evident that 1 week in the refrigerator was adequate for maximum precipitation. With material from hog 29, no difference in capacity to precipitate Type XIV antibody was found after the solution had remained in the refrigerator for 6 weeks.

The same type of variation in capacity to precipitate with Type XIV antiserum was observed with blood group A substance from human saliva and stomach (Table II) as well as with materials prepared by the same methods from O saliva and stomach. Data are included on the relative viscosity and on the capacity of the various samples to precipitate anti-hog A formed in man (7). For instance, the substances from human stomachs 2 and 3 both showed

the same capacity to precipitate anti-A but that from stomach 2 was about twice as potent in precipitating antibody from Type XIV antipneumococcal serum. Similarly, the products from B. K. precipitated considerably more antibody in the cross-reaction than did A. B.$_4$ or W. H.$_1$ 10 per cent precipitates

TABLE I

Cross-Reaction of Purified Blood Group A and O Substances from Individual Hog Stomach Linings with Type XIV Antipneumococcal Horse Sera

Amount blood group substance added	Total nitrogen precipitated																
	Blood group A substances										Blood group O substances						
	Hog 3	Hog 8	Supernatant + Hog 8	Supernatant + Anti-body	Hog 10	Supernatant + Hog 10	Supernatant + Anti-body	Hog 15	Hog 16* (a)	Hog 16* (b)	Hog 13	Hog 19	Hog 25	Hog 27	Hog 29‡ (a)	Hog 29‡ (b)	Hog 29‡ (c)
μg.	μg.	μg.			μg.			μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
0.5 ml. H635																	
50	15	24	++±	+	13	++	++	8	12	9	16	12	7	24	23	21	18
100	22	32	++	+	8	++	++	13	18	15	27	20	8	33	28	29	25
150	31	42	++	+	13	++	++	17	24	19	39	24	14	46	35	36	31
250	46	54	++	+	16	++	++	23	30	25	43	23	18	64	44	46	39
500	72	72	+±	+±	19	++	++	35	39	38	64		23	80	63	66	60
0.5 ml. A66																	
50		19	++++	+	16	++	+						17	31			
100		25	++++	+++	20	++	+±						24	48			
150		28	+++	+++	20	+	+±						31	54			
200		33	+++	+++	21	+±	++						32	77			
300		36	+++	+++									37	73			
400		42			17								43	96			
500					18	+±	++										
η rel.§ (5)...	1.65	1.39			1.71			1.64		1.58	1.58	1.27	1.56	1.40		1.65	

* Set (*b*) was identical with set (*a*) but tubes were allowed to remain in the refrigerator for 2 weeks instead of 1 week.
‡ Sets (*b*) and (*c*) run 6 weeks after set (*a*), set (*c*) was washed and analyzed after 2 weeks in the refrigerator. Fresh solution used for set (*a*).
§ Relative viscosity of 0.2 per cent solution in 0.9 per cent saline.

(7) although they showed only about one-half the potency in precipitating anti-A.

The preparations from amniotic fluid and from human stomach 1 were unusually potent in precipitating with Type XIV antiserum although they showed only 17 per cent of the capacity of other samples in precipitating anti-A. Blood group B substance from human saliva also precipitated with Type XIV antiserum, essentially similar data being obtained with the phenol-insoluble and 10 per cent precipitate.

TABLE II

Cross-Reaction of Purified Blood Group Substances from Human Sources with Type XIV Antipneumococcal Horse Sera

Amount substance added	Total nitrogen precipitated																					
	Human saliva															Amniotic fluid	Human stomach					
	A substance												B substance		Substance from O saliva		A substance	A substance				O substance
	A. B_4	G. C.		W. H.$_1$		W. H.$_2$		B. K.		M. S.	W. G.		S. E.		F. P.	Bd		1	2	3	4	5
	10% ppt.	Phenol-insol.	10% ppt.	Phenol-insol.	10% ppt.	Phenol-insol. undigested	10% ppt.	Phenol-insol.	10% ppt.	Phenol-insol.	Phenol-insol.		Phenol-insol.	10% ppt.	10% ppt.	10% ppt.	Phenol.-insoluble					
μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
0.5 ml. H635																						
50	19	18		18	15	10	16	20	16	31	22		20	12	30	11	44	22	18	11	17	30
100	30	26		30	23	20	29	32	29	47	38		31		47	16	70	37	33	16	22	45
150	34	36		39	29	28	39	46	41	61	46		36		62	25	86	45	38	18	27	56
250	46	50		50	43	40	60	63	60	74	61		42	46	86	29	108	52	46	23	39	72
500	61	60		81	55	58	102	86	81	88			56	65	105	42	125	65	62	29	50	
0.5 ml. A66																						
50		17	18		16								14	9			27					
100		25	29		25								17	14			43					
150		36	35		34								20	16			51					
250													26	24			63					
500					40								37	33			88					
η rel (7)...	1.25	1.23	1.25	1.35	1.23	1.13	1.13	1.27	1.13								1.20		1.11		1.13	
Relative capacity to precipitate anti-A (7).....	100	90	100	78	100	25	80; 100	48	46	86	40						17	17	100	100	63	

DISCUSSION

The data presented confirm previous observations (3) that purified hog blood group A substances precipitate with Type XIV antipneumococcal horse serum and in addition establish that hog blood group O substances and human blood group substances from individuals of groups A, B, and O also cross-react with Type XIV antipneumococcal antibody. Since these purified preparations appear to contain some of the essential constituents of the Type

XIV specific polysaccharide, *N*-acetyl-*d*-glucosamine and galactose, it seems reasonable to attribute the cross-reactivity to this similarity in chemical composition and thereby to provide a reasonable explanation for the capacity of Type XIV antiserum to agglutinate human erythrocytes of all four blood groups. Preparations of the various blood group substances also inhibit the agglutination of human A, B, or O erythrocytes by the Type XIV antiserum.

The broad zones over which supernatants from the reaction of the blood group substances with Type XIV horse antiserum invariably showed the presence of both antigen and antibody are also best interpreted on the basis of a cross-reaction similar to that between Types III and VIII antipenumococcal horse antibodies and their heterologous specific polysaccharides (10, 11).

Individual preparations of purified hog blood group A substances, all of which were shown by quantitative immunochemical assays to be of equal potency in their reactivity with anti-hog A formed in man (5), vary widely in their cross-reactivity with Type XIV antipneumococcal antibody (Table I). Similar variations apparently unrelated to the capacity of the substance to precipitate anti-A were also found for the human A substances from saliva and stomach (7). Whether the differences in the cross-reactivity of the various O substances from hog stomachs and of materials of similar composition from human group O saliva are also unrelated to their potency as group O substances cannot yet be decided since quantitative precipitin assays for anti-O are not available and assays by inhibition of hemagglutination are not sufficiently precise to provide a definitive answer. The differences in cross-reactivity among the individual A or O preparations could not be correlated with the relative viscosity of their solutions, and do not appear to be due to technical difficulties such as failure to attain maximum precipitation since identical results were obtained when precipitin analyses were (hogs 16 and 29, Table I) carried out after the tubes had been in the refrigerator for 2 weeks instead of 1 week; in addition no detectable change in precipitating capacity of the substance from hog 29 occurred after the solution had remained 6 weeks in the refrigerator.

It is not possible at present to offer an adequate explanation of these findings. However, it has previously been established by quantitative precipitin assays that solutions of hog blood group A substance are extraordinarily stable with regard to their blood group A activity, no detectable reduction in potency occurring after exposure for 2 hours at 100°C. in solutions varying in pH from 2.97 to 7.58 (12). That such individual preparations may vary in their cross-reactivity with Type XIV antipneumococcal serum and not in their blood group A activity suggests that the two activities may be associated with different portions of the complex molecule and that it may be possible under suitable conditions to alter one without affecting the other. It is apparent that any

adequate explanation of the chemical basis for the unique biological activities of the blood group substances must account for the differences herein noted.

SUMMARY

Purified blood group A, B, and O substances from hog and human sources precipitate with Type XIV antipneumococcal horse serum and provide an explanation for the observation that Type XIV antibody agglutinates human erythrocytes of all four major blood groups.

Individual preparations of A substance or O substance from either species vary in their capacity to precipitate Type XIV antibody although the hog A substances did not differ in potency toward anti-A. Similarly, no correlation between A activity and reactivity with Type XIV antibody could be found among the human A substances.

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THE NATURE OF THE VIRUS RECEPTORS OF RED CELLS

I. Evidence on the Chemical Nature of the Virus Receptors of Red Cells and of the Existence of a Closely Analogous Substance in Normal Serum

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The ability of red cells from various species to adsorb and elute the viruses of influenza, mumps, and Newcastle disease is of general interest in the problem of the mechanism of infection by viruses since the red cell-virus system may well involve principles which apply to the virus-host cell relationship. The elution of virus from cells, accompanied by the disappearance of the virus-adsorbing capacity of the cells, indicates that an active destructive process has occurred. It has been suggested (1) that the destruction of receptors is due to an enzyme possessed by the virus, which is active against a substrate possessed by the cell, and no subsequent developments have suggested a more likely alternative. The ultimate goal in proof of this theory is clearly to isolate the cellular substrate and show what type of chemical action the virus may have upon it.

There are several main ways in which the problem may be attacked: (1) attempts to isolate the receptor from the red cell; (2) treatment of the intact cell with reagents which may inactivate the receptor and give some clew to its chemical nature; and (3) searching for analogues of the receptor substance from other biological sources. In the present work only the second and third methods are used.

Methods

The virus strains used in this work were the PR8 strain of influenza A, the Lee strain of influenza B, and strain L230, which is an influenza A strain isolated in the epidemic of early 1947. The virus suspensions used were all prepared from allantoic fluid and were usually dialyzed against phosphate buffer before use. All of the hemagglutinin and agglutinin inhibition titrations were carried out by the densitometric method of Hirst and Pickels (2) and titers are expressed as the reciprocal of the final dilution of either virus or serum. All titration values have been corrected and given in terms of initial serum or virus concentrations so that they may be directly compared.

EXPERIMENTAL

The Treatment of Red Cells with Physical and Chemical Agents and Their Effect on the Receptor Substance

Red cells can be exposed to various reagents and tested for their capacity to agglutinate with different quantities of virus but this technique has deficiencies owing to the frequent occurrence of spontaneous red cell agglutination after

treatment, which renders the test unworkable. Another method of testing the activity of receptors is to measure the amount of virus which treated red cells can absorb from a virus suspension under standard conditions and to compare this with the adsorptive capacity of various concentrations of untreated red cells. By this method the degree of receptor damage is measured in terms of receptor-virus interaction and the purely secondary effect of agglutination is excluded. On comparing the adsorption of treated cells with that of equal and lesser concentrations of normal cells one can estimate in a rough quantitative way the degree of receptor damage.

In the following series of experiments fowl red cells were treated in various ways and, after washing to get rid of the excess of reagent, the cells were tested for their capacity to adsorb virus by mixing them with influenza suspensions at 0°C. for 30 minutes. The cells were centrifuged out and the supernatant titered for residual unabsorbed hemagglutinin(1). At the same time adsorption was carried out with normal cells, both in the same concentration as the treated cells and in lesser amounts in order to provide a scale of comparison. An example of the change in virus adsorption with various concentrations of cells is given in Table IV. With a relatively high concentration (1.5 per cent) adsorption is usually fairly complete and falls off little as the concentration of cells is reduced. With very dilute cell suspensions the adsorption is so small that it cannot be accurately measured in the presence of the large amount of residual virus; but there is a wide range where satisfactory values are obtained.

The most serious drawback to a clear interpretation of the results of this type of experiment is that the reagents used may cause damage to the cell which reduces its adsorption capacity but this does not necessarily mean direct damage to the receptor. Under certain conditions cells or cell debris may adsorb virus in such a manner that it cannot be readily eluted and it is probable that such adsorption is due to substances other than the surface virus receptor. In all tests reported below, complete or nearly complete elution of virus was demonstrated after adsorption on treated cells, though the data are not given in the tables.

Effect of Hemolysis on Virus Receptors

Red cells were hemolyzed in distilled water and washed thoroughly in saline until free of red color. This treatment results in a considerable change in morphology of the ghosts. Various concentrations of this stromal material and of untreated cells were added to a suspension of PR8 virus and their adsorptive capacity was tested (Table I). The stroma definitely adsorbed (and eluted) virus but had only about one-twentieth of the capacity of untreated cells. Similar results were obtained with stroma prepared by homogenization of cells in saline.

In another experiment red cells were hemolyzed with saponin (toxic) (1 cc. of a 5 per cent aqueous solution per 4 cc. of packed cells) and the ghosts were washed free of visible hemoglobin. In this preparation the morphology of the cells remained intact and their capacity to adsorb virus was undiminished (Table I) and when these ghosts were treated with 5 per cent formalin for 2 days and then washed there was also no effect on their adsorptive capacity. When the cell ghosts were homogenized their adsorptive capacity dropped markedly as in the first experiment detailed. Repeated washing of the stroma does not further reduce the adsorptive capacity.

TABLE I

Adsorption of Virus (PR8) by Red Cells Lysed with Distilled Water and Saponin

	Adsorption with normal cells at various concentrations*				Control no cells	Adsorption with water-lysed stroma at various concentrations*				
	3 per cent	1.5 per cent	0.75 per cent	0.37 per cent		80 per cent	40 per cent	20 per cent	10 per cent	5 per cent
Titer after adsorption.....	6	11	20	39	91	4	9	16	32	79

Adsorption with:

	3 per cent normal untreated	Control no cells	Saponin* ghosts 3 per cent	Saponin ghosts* in 5 per cent formalin 3 per cent	Saponin ghosts* homogenized 3 per cent
Titer after adsorption.....	74	294	74	60	208

Red cells or stroma and virus were combined in equal volume at 0°C. for 30 minutes and after centrifugation the supernatant hemagglutinin titer was tested.

* Concentration expressed in terms of red cells before lysis.

The disappearance of receptor activity when cells are broken up seems to correlate nicely with the findings of Friedewald, Miller, and Whatley (3) who demonstrated the presence of an inhibitor in the supernatant fluid when cells were homogenized, which inhibitor they believe to be the receptor substance. However, if red cells are treated with saponin and the supernatant is dialyzed free of saponin it can be shown to contain a considerable amount of virus inhibitor when tested by Friedewald's technique. Nevertheless, the ghosts from which the inhibitor has been extracted show no decrease in their ability to adsorb virus, indicating either that only a small fraction of the receptors has gone into solution or that some of the extractable inhibitor is of a different character from the virus receptor.

Resistance of Receptors to Heating

Saline suspensions of 3 per cent red cells were heated at temperatures of 56°C. and over for 30 minutes and their ability to adsorb virus was tested

together with that of control cells (Table II). The adsorptive capacity of cells heated at 56° and 65°C. did not change detectably, while at higher temperatures specific adsorption (and elution) was found but it was less than 12 per cent of normal and there were considerable hemolysis and breakdown of cells. Since the reduced adsorption after using higher temperatures may have been due to

TABLE II

Adsorption of Virus (PR8) by Red Cells and Water-Lysed Stroma after Heating at Various Temperatures

	Whole cells								
	Untreated cells at various concentrations				Control no cells	3 per cent cells heated for 30 min. at various temperatures			
	3 per cent	1.5 per cent	0.75 per cent	0.37 per cent		56°	65°	75°	85°
Titer after adsorption.....	12	20	52	64	182	4	3	97	102
	Water-lysed stroma								
	Unheated, at various concentrations*				No stroma	60 per cent stroma* heated 30 min. at various temperatures			
	60 per cent	30 per cent	15 per cent	7.5 per cent		56°	65°	75°	85°
Titer after adsorption.....	20	39	74	120	182	13	20	39	49

Red cells (heated and unheated) were combined in equal volume with PR8 virus (titer 182) and after 30 minutes at 0° C. the cells were removed and the hemagglutinin titer of the supernatant determined.

* All percentages are in terms of normal cells before lysis.

structural alteration in cells rather than receptor inactivation, a similar experiment was done with cell stroma obtained by hemolysis with water, in which the morphology was already altered, and with this preparation heating at 85°C. resulted in a loss of only a little over half the activity. It is clear that the receptor substance is quite stable to heat.

Stability of Receptor Substance at Different pH Levels

PR8 virus was suspended in citrate-phosphate buffer solutions at different pH levels and after 30 minutes dilutions of the suspensions were made in phosphate buffer (pH 7.1, M 0.11) and the hemagglutinin titered. As shown in Table III there was no adverse effect on the hemagglutinin after suspension in solutions of pH 2.4 to 10.3. Red cells were then suspended in similar buffers covering the same range of pH and after 30 minutes the cells were washed in

phosphate buffer at pH 7.1 and were then added in 3 per cent concentration to equal volumes of PR8 virus which had a titer of 270. All of the cells adsorbed virus well except those which had been exposed to a pH of 3.14 and 2.60, the last adsorbing practically no virus. Hemolysis occurred at these lower pH levels, indicating that the effect may have been due to alteration of the cells and not necessarily the receptor substance.

Effect of Tryptic Digestion on the Receptor Substance

Tryptic digestion of cells was usually accompanied by some destruction of cell morphology and ordinarily by complete loss of receptor activity. Saponin-

TABLE III

Effect of Exposure at Different pH Levels on the Influenza Virus Hemagglutinin and on Red Cell Receptors

Virus exposed 1 hr. at:	Titer	Red cells exposed 1 hr. at:	Titer of supernatant after adsorption
pH		*pH*	
2.42	256	2.60*	208
3.06	239	3.14*	32
4.12	158	4.19	20
4.78	158	4.85	20
5.40	158	5.50	24
6.11	138	6.21	16
7.10	158	7.10	20
10.33	158	10.15	24
		Control no cells	275

* Cells showed some hemolysis after treatment.

lysed cells treated with formalin, however, retained their morphology on digestion with crystalline trypsin but this procedure was accompanied by a marked though not quite complete loss of virus-adsorbing capacity. From this latter result it seemed probable that trypsin had a direct effect on the receptor.

The extraction of cells and stroma with lipid solvents resulted in complete loss of adsorbing capacity but the change in the physical state of the extracted materials was such that no conclusions could be drawn.

The Effect of Oxidizing Agents on the Receptor Substance

In casting about for reagents which had some degree of specificity against various classes of natural substances the effect of sodium periodate was tested on normal red cells.[1] This substance even in low concentration was very effective in inactivating the receptor substance without destroying the mor-

[1] The author is indebted to Dr. R. D. Hotchkiss of The Rockefeller Institute for Medical Research for this suggestion.

phology of the cell. In order to try to establish the specificity of this effect various other oxidizing agents were tested (Table IV). Equal volumes of 1.5 per cent red cells were added to these reagents in several strengths and after standing 1 hour the cells were spun out, washed once in M/20 sodium thiosulfate

TABLE IV

Effect of Oxidizing Agents on the Adsorbing Capacity of Red Cells

Oxidizing agent	Cells treated with oxidizing agent in various concentrations						Untreated cell controls		
	M/10		M/100		M/1000				
	Titer after adsorption	Per cent of normal adsorbing capacity	Titer after adsorption	Per cent of normal adsorbing capacity	Titer after adsorption	Per cent of normal adsorbing capacity	Concentration normal cells	Per cent of full concentration	Titer after adsorption
							per cent		
$K_3(Fe(CN))_6$	3.5	90	2.8	100	5.7	75	1.50	100	3.0
$KMnO_4$	42*	18	14*	48	18*	45	1.35	90	3.5
$K_2Cr_2O_7$	21*	42	3	100	4	79	1.20	80	3.8
I_2	4	79	3.7	83	4	79	1.05	70	8.6
H_2O_2	4	79	4.3	78	4.3	78	0.90	60	11
$NaIO_3$	3.5	90	3.2	95	3.0	100	0.75	50	12
$NaIO_4$* glucose	3.5	90	3.7	83	3.7	83	0.60	40	23
$NaIO_4$	97	3	97	3	112	0	0.45	30	25
							0.30	20	37
							0.15	10	64
							0.075	5	79
							0.037	2.5	104
							Control no cells	—	109

Equal volumes of cells (1.5 per cent) and oxidizing reagent were combined for 1 hour after which the cells were centrifuged and the supernatant discarded. The cells were washed once with sodium thiosulfate M/10 and twice with saline. The cells were restored to original volume, added to an equal quantity of PR8 virus (titer 109) at 0°C., kept in suspension for 30 minutes, removed by centrifugation, and the supernatant was titered for hemagglutinins.

* Cells showed marked morphological changes after treatment.

solution and twice in saline. They were tested for their capacity to adsorb influenza virus as compared with normal cells. Except where the reagents caused morphologic changes in the cells their adsorbing capacity was reduced only 25 per cent or less. Considering the very minor changes in adsorption with the control cells in this range a loss of 25 per cent cannot be considered significant. In marked contrast was the effect of sodium periodate which abolished receptor activity in thousandth molar concentration without any

change in cellular morphology although there was a change in hemoglobin color and slight hemolysis. Sodium iodate had no effect and neither did periodate to which glucose had been previously added. This would point to a specific oxidizing effect of the periodate radical which is known to affect mainly carbohydrates in biological materials by splitting the carbon-carbon bond where there are adjacent hydroxyl groups.

On the basis of the evidence presented one may say that at least part of the active agent responsible for the adsorption of virus to red cells may be carbohydrate, possibly polysaccharide.[2] The stability of the receptor to alkali and to heat would be in keeping with this suggestion while the destruction of receptor activity by trypsin indicates that a protein component may be present and necessary for activity.

The Nature of the Inhibitor Substance in Serum

When the inhibition of agglutination of red cells by influenza virus was first used for the titration of specific antibody (5) it was discovered that the normal sera of rabbits and ferrets, though free from antibody, nevertheless had a fairly high titer of inhibition, so that when normal sera were combined with virus the latter could be prevented from agglutinating red cells even in fairly high dilution of serum. This *in vitro* inhibition of agglutination is not paralleled by *in vivo* neutralization except with the influenza A strains of 1947 (6). The inhibitory titer varies from animal to animal and is very high in rabbit and ferret sera but low in horse serum. The inhibitor is extremely stable at high temperatures and resists 100°C. for 15 minutes. This finding does not accord with that of McCrea (7) who reported inactivation of rabbit serum inhibitor at 62°C. Soon after the discovery of the effect of periodates on the receptors of red cells it was found that they had a similar destroying action on the inhibitory substance in normal serum (Table V) which suggested that serum inhibitor might be related to the red cell receptor.

One volume of serum (inactivated at 56° for 30 minutes) was added to one volume of 0.05 M $NaIO_4$ and after 2 hours the remaining periodate was inactivated by the addition of glucose (two volumes of 5 per cent solution). Rabbit sera containing specific influenza antibodies were treated in a similar manner. Sera which were treated with sodium periodate which had been previously inactivated with glucose, and untreated controls were tested together with periodate-treated sera for their inhibitory titer with 4 units of PR8 virus. The inhibitor in normal rabbit serum was reduced by sodium periodate from a titer of 145 to less than 16, while inactivated sodium periodate had no effect.

[2] The preliminary observations on the effect of periodate on receptors were made while the author was on the staff of the International Health Division of The Rockefeller Foundation and a brief summary, including the implication of carbohydrate, was published in the annual report of that Division and was made generally available in late 1946 (4).

The immune rabbit serum was somewhat reduced in titer by sodium periodate. Similar results were obtained with an acute and convalescent pair of sera from human influenza A infection, with a marked drop in the acute instance and a lesser percentage drop in the inhibitory titer of the convalescent serum.

Action of Oxidizing Agents and Trypsin on Serum Inhibitor

The same oxidizing agents which were shown to be ineffective in inactivating the virus receptor on the red cell were tested on the inhibitor of normal rabbit serum. Equal volumes of reagent and serum were combined and after 2 hours were dialyzed against a phosphate buffer. HCl was added to another specimen until the pH dropped to 2.0. After 24 hours, dialysis, inhibition tests were

TABLE V

Effect of Sodium Periodate on Agglutination Inhibition with Normal and Immune Sera as Tested with PR8 Virus

Treated with	Normal rabbit serum	Immune rabbit serum	Acute influenza A human serum	Convalescent influenza A human serum	Increment of rise in human serum
Control	145	295	111	680	6.1 times
$NaIO_4$ and glucose	137	335	111	580	5.2 times
$NaIO_4$	<16	208	16	240	15 times

Reagents (saline, $NaIO_4$ inactivated with glucose, and $NaIO_4$ M/20) were added to serum in equal volume. After 30 minutes two volumes of glucose were added to the sera receiving periodate. Hemagglutinin inhibition titrations were done with 4 units of PR8 virus.

performed against 4 units of PR8 virus. The results (Table VI) were clear cut and showed no major effect on the inhibitor by any oxidizing agent except sodium periodate, which correlates well with the similar experiment on red cell receptors (Table IV). A pH of 2.0 had no effect on the inhibitory titer, which is consistent with the red cell effect only if one interprets the loss of receptor activity from cells at low pH as being due to change in cell morphology.

Normal rabbit serum was treated with an equal volume of crystalline trypsin (250 mg. per cent) at 37°C. for 3 hours with a marked reduction in inhibitory titer as shown in Table VII, an effect which is consistent with the conclusion reached with respect to the effect of trypsin on formalinized red cell ghosts. Lipid extraction did not lower the inhibitory titer of serum.

The analogy of the normal serum inhibitor and the red cell receptor is, on the whole, rather striking in that they both undergo a similar destruction with sodium periodate but not with other oxidizing agents, both are destroyed by trypsin, and both are quite stable to heat and to high pH levels. A more clinching argument for their similarity would be found if it could be shown that the inhibitor is destroyed by virus.

Effect of Influenza Virus on Normal Serum Inhibitor

If influenza virus can destroy the inhibitor in normal rabbit serum then a titration of normal serum which has been in contact with virus for some time

TABLE VI

Treatment of Normal Rabbit Serum with Oxidizing Agents and Its Effect on Inhibition of Agglutination

Serum treated with:	Hemagglutinin inhibition titer after treatment with reagent in various concentrations	
	0.1 M	0.01 M
$K_3(Fe(CN))_6$	275 M	223 M
$KMnO_4$	147 M	169 M
$K_2Cr_2O_7$	362 M	275 M
I_2	223 M	182 M
H_2O_2	239 M*	294 M*
$NaIO_4$	<8 M‡	64 M
HCl	275 M§	
Control	294 M	

Reagents were added in equal volume to undiluted rabbit serum which had previously been inactivated at 56°C. After 1 hour the mixtures were dialyzed against a phosphate buffer and inhibition tests were carried out in the usual manner against 4 units of PR8 virus. The titrations were not very exact because of volume changes which took place on dialysis, which were not corrected.

* Concentration added was 0.88 M.

‡ Concentration added was 0.05 M.

§ Concentration sufficient to bring to pH 2.0, pH was 6.97 after dialysis.

TABLE VII

Inhibition of Agglutination by Serum Treated with Crystalline Trypsin

	Tested with:	
	PR8 Virus	Lee virus
Serum control	60	42
Serum treated with trypsin	<16	<16

Equal volumes of serum and trypsin solution (250 mg. per cent) were combined and incubated at 37°C. for 3 hours.

should give less inhibition on the addition of the test red cells than a similar titration in which the virus had been added to the serum immediately before the addition of cells. When virus was left in contact with serum dilutions for 20 hours or longer very definite decreases in serum inhibition were noted. Very definite decreases in serum inhibition have also been found over short periods of time but involve other techniques which will be described in a later paper.

In the first experiment (Fig. 1) the L230 strain was used. Normal rabbit serum, inactivated at 56°C. for 30 minutes, was diluted serially in saline. To one set of serum dilutions virus was added so that the final concentration in each test tube would be 18 units. These tubes stood for 20 hours at room temperature. After 20 hours the same amount of virus was added to a second set of

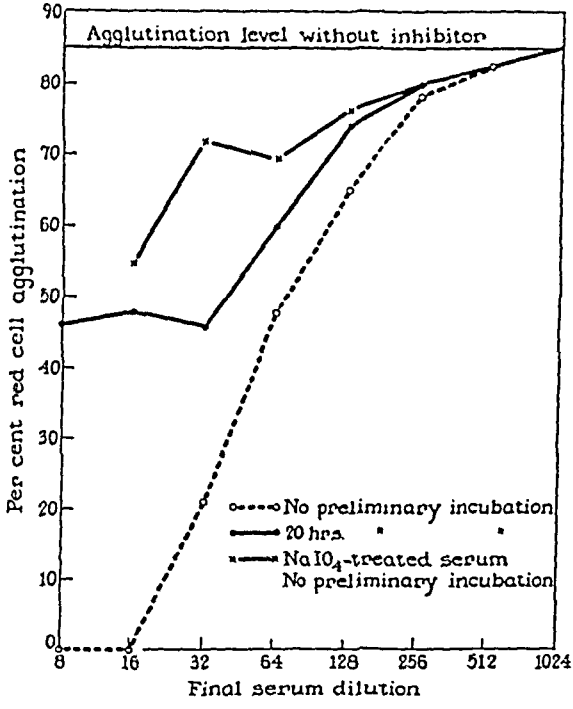

FIG. 1. Agglutination inhibition test with strain L230 virus and normal rabbit serum. In the case of the middle curve (solid line) the virus was added to the serum 20 hours before the red cells. For the lower curve the virus was added 10 minutes before testing. The upper curve represents the results with serum treated with $NaIO_4$ and tested with virus added 10 minutes before testing with red cells. The curves show the percentage of the red cells added which were agglutinated by the virus and sedimented in 75 minutes time. The amount of virus used sufficed to agglutinate 85 per cent of the cells in the absence of any inhibitor.

serum dilutions and after 10 minutes cells were added to the entire test, which was then read in 75 minutes. The percentage of cells agglutinated is plotted for each tube. The amount of virus used was sufficient in the absence of inhibitor to agglutinate 85 per cent of the red cells, but this maximum of agglutination could have been achieved by one-fourth as much virus. The strain used is one which normal sera inhibit to exceptionally high titer and it is even neutralized by normal rabbit sera in *in ovo* tests (6). When this virus was added to normal rabbit serum just before the addition of the indicator cells there was complete suppression of agglutination in dilutions of serum 1:8 and 1:16, and detectable inhibition was present at a dilution of 1:512. When virus and

serum were allowed to stand in contact for 20 hours however, serum-virus mixtures in the lower range of serum dilution showed a marked increase in the agglutination of red cells, from zero to 48 per cent and this increase was significant in dilutions as high as 1:128. Similar results with the Lee strain are shown in Fig. 2. In each case control virus hemagglutinin titrations showed no change in titer over the 20 hour period. For comparison periodate-treated sera are included in each figure, the virus being added in these titrations immediately before the cells.

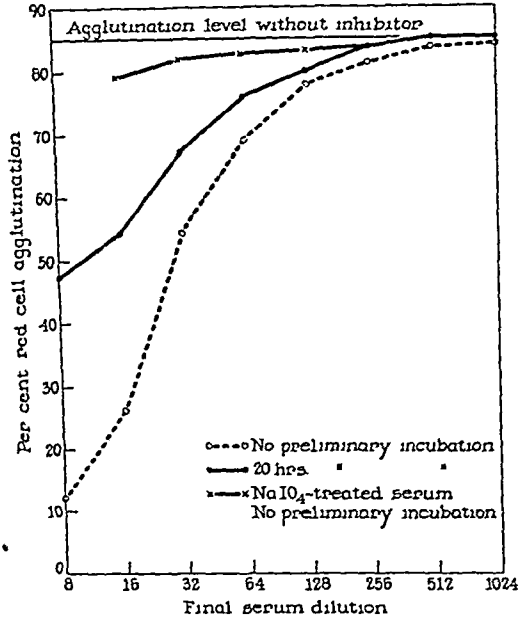

FIG. 2. Agglutination inhibition test with the Lee strain and normal rabbit serum. See legend of Fig. 1.

Contrary to the usual result when an active agent and an inhibitor are allowed to incubate in contact, the virus-inhibitor system showed a decrease in inhibition as virus suspension and inhibitor were allowed to interact. The simplest interpretation of this is that the virus has the ability to destroy the inhibitor in serum. It is possible that the inhibitor is destroyed by an agent present in allantoic fluid not directly connected with the virus. This possibility seems unlikely in view of the analogies of the reaction with that of the red cell-virus system in which the active agent is generally accepted to reside on the virus. Normal allantoic fluid and allantoic fluid from which most of the virus has been removed by centrifugation, do not remove the inhibitory substance from serum while the inhibitor-destroying property is retained by virus purified by centrifugation or by adsorption on, and elution from red cells.

Burnet, McCrea, and Stone (8) have shown that a cholera vibrio filtrate has the power to remove receptors from red cells in a manner quite analogous to the action of viruses. During the course of this investigation Burnet *et al.* (9) also reported the inactivation of serum inhibitor with cholera vibrio filtrates and by influenza virus but no details of method for the latter were offered. The main facts concerning the cholera vibrio filtrate have been confirmed in this laboratory.

DISCUSSION

The discovery that influenza virus after adsorption on red cells or respiratory cells (1, 10) elutes readily and completely, suggested strongly that the virus is possessed of an enzyme which is capable of inactivating a receptor substance on red cells. Since that time no contrary hypothesis has been put forward to explain the initial facts and most investigators of the problem have directed their efforts toward tests of this theory. Proof of it very clearly consists in identifying the substrate and the chemical nature of the enzymatic destruction of it by virus.

The present work suggests strongly that the substrate in question is mucopolysaccharide in nature and that both the protein and the polysaccharide components are necessary for its combination with virus. It seems very likely that a close analogue of this receptor substance is present in considerable quantity in normal serum.

The basic facts concerning the action of periodate and the implication of carbohydrate in the receptor substance, published in 1946 (4), suggested the exploration of mucins to Burnet and his collaborators (11). Very recently Burnet (12) has stated, in a preliminary note, that fluid from a pseudomucinous cyst and blood group O substance inhibit influenza virus agglutination markedly, and that the inhibition is reduced by incubating virus and O substance together, probably in a manner analogous to the serum inhibition tests shown here. This is a long step toward the solution of the problem and is completely in keeping with the implications in this paper.

Greene and Woolley (13) have found several naturally occurring polysaccharides (most notably apple pectin) to have a pronounced effect both in inhibition of red cell agglutination by influenza virus and in prevention of infection in chick embryos. Later Ginsberg, Goebel, and Horsfall (14) found that the capsular polysaccharide of type B Friedländer bacillus blocked the agglutination of red cells by mumps virus but not by influenza A or B. Both groups found that red cells could be treated with carbohydrate and then washed and still retain their inagglutinability. The action in these cases seems to be between carbohydrate and red cells, with a specific blocking of receptor points. The demonstration of action between virus and carbohydrate was not convincing and with apple pectin in higher concentrations the red cells were

actually agglutinated by the carbohydrate. These experiments, though highly interesting, do not suggest that the polysaccharides used were necessarily chemical analogues of the receptor substance. Immersion of red cells in serum containing inhibitor, for example, does not destroy their agglutinability by virus if they are washed before testing, and they do not adsorb inhibitor from the serum.

Both Friedewald, Miller, and Whatley (3) and Bovarnick *et al.* (15, 16) have reported the extraction from cells of virus inhibitors which are destroyed in the presence of virus and which they believe may be the receptor substance in solution. The latter group obtained potent extracts by the use of lipid solvents and their best material contained about 50 per cent polysaccharide. The presence of polysaccharide is in keeping with the work reported above. There is a striking resemblance of the virus receptor substance to the blood group substances in that the form in the cell seems to be water-insoluble while water-soluble forms can be found elsewhere.

The destruction of inhibitor in sera by periodate suggests a practical use for this agent in eliminating this factor from sera which are to be tested for influenza antibody. This aspect of the inhibitor problem will be elaborated in another paper.

SUMMARY

The influenza virus receptors of fowl red cells and the influenza virus inhibitor of normal rabbit serum have the following attributes in common: They are stable at high temperatures and in solutions of pH as high as 10.0. They both resist destruction by a number of oxidizing agents but are readily destroyed by sodium periodate, trypsin, and influenza virus. These facts suggest that the red cell receptor and the normal serum inhibitor are either the same or analogous substances and that they may belong to the mucoprotein class of compounds.

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THE NATURE OF THE VIRUS RECEPTORS OF RED CELLS

II. The Effect of Partial Heat Inactivation of Influenza Virus on the Destruction of Red Cell Receptors and the Use of Inactivated Virus in the Measurement of Serum Inhibitor

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In a previous paper (1), it was shown that the influenza virus receptors of red cells and the virus agglutination inhibitor present in normal serum have many features in common. They are both destroyed by trypsin, sodium periodate in high dilution, and by influenza virus itself. They are both quite heat-stable and resist exposure to wide changes in hydrogen ion concentration. Of these similarities, the most striking and significant is the destruction by influenza virus. In the present paper, the adsorption and elution of influenza virus after heating at 56°C. will be described, together with the use of heated preparations for the measurement of serum inhibition. This method of measuring serum inhibition is such a great improvement over the use of unheated virus that the destruction of serum inhibitor by active virus can be much more conclusively demonstrated.

Methods

The strains used in the present experiments were the PR8 strain of influenza A, the Lee strain of influenza B, and strain L230 which was isolated from the influenza A epidemic of 1947 and is characterized by the fact that it is inhibited by high dilutions of normal serum. Hemagglutinin titrations and serum inhibition tests were performed in the usual manner by the method of Hirst and Pickels (2). All the rabbit serum used was from a single pool and, unless otherwise indicated, was inactivated by heating at 56°C. for 30 minutes before use. All of the dilutions of serum were prepared in excess amounts so that they could be put into the final tubes without blowing out the pipettes. Pipettes were changed with each dilution. All titers are expressed as the reciprocal of the dilution and have been corrected for volume changes so that they can be compared directly. The hemagglutinating activity is occasionally referred to in terms of units. A unit may be defined as the amount of hemagglutinin which, in a final volume of 2 cc., will cause 50 per cent of the cells to agglutinate. The titer of a preparation (expressed as the reciprocal of dilution) is therefore the number of units contained in 2 cc. of the suspension.

EXPERIMENTAL

It was observed by Francis (3) that Lee (B) virus, when heated at 56°C., lost little of its hemagglutinin titer but gave higher inhibition titers with human sera than did unheated virus in equivalent amounts. The effect was especially

noticeable in sera with low antibody levels. Although Francis was mainly concerned with the explanation of the phenomenon in terms of antibody measurement, it seemed very striking that a most marked effect was obtained with normal animal sera which contained no specific antibody. This suggested that the underlying mechanism of the differences in inhibition might lie in a change in the properties of the hemagglutinin itself, and to test this possibility the following experiments were performed.

TABLE I

Effect of Heat on Adsorption and Elution of Influenza Virus from Red Cells

Strain		Titer	Heated at 56°C. for					
			0 min.	5 min.	10 min.	15 min.	30 min.	60 min.
Lee(B)	1	Before adsorption	104	74	64	69	64	69
	2	Supernatant after adsorption	13	6	6	6	5	5
	3	Units of virus adsorbed	91	68	58	63	59	64
	4	Eluted virus	84	49	37	24	2.5	<2
	5	Adsorbed virus eluted, *per cent.*	92	72	64	38	4	<3
PR8(A)	1	Before adsorption	169	138	104	112	84	56
	2	Supernatant after adsorption	37	52	42	37	20	16
	3	Units of virus adsorbed	132	86	62	75	64	40
	4	Eluted virus	104	45	39	37	30	14
	5	Adsorbed virus eluted, *per cent.*	79	52	63	49	47	35
L230(A)	1	Before adsorption	60	60	52	49	37	26
	2	Supernatant after adsorption	5	6	6.5	4	4	4.6
	3	Units of virus adsorbed	55	54	48.5	45	33	21.4
	4	Eluted virus	45	42	42	42	24	<2
	5	Adsorbed virus eluted, *per cent.*	82	78	86	93	73	<9

Effect of Heating on the Spontaneous Elution of Influenza Virus from Red Cells

Influenza virus of three strains (Lee (B), PR8 (A), and L230) was heated at 56°C. for intervals up to 1 hour and the samples were tested for hemagglutinin titer (Table I, line 1). In each case, the heating caused a progressive decrease in titer. To each sample an equal volume of 1.5 per cent red cells was added at 0°C. and the mixture left for 30 minutes at this temperature. The cells were removed by centrifugation and the residual unabsorbed virus of the supernatant was tested (Table I, line 2). The centrifuged cells were then suspended in a volume of saline equal to that of the original virus sample and incubated for 3 hours at 37°C. The cells were again removed and the eluted virus in the supernatant titered.

The results with the Lee virus were the most striking. Heating this strain at 56°C. for 1 hour resulted in a 30 per cent drop in titer. The hemagglutinin in the various specimens was almost equally well adsorbed on red cells. Ninety-four per cent of the unheated virus eluted (Table I, line 3), while with

progressive heating the elution became less and less marked, and with virus heated for 1 hour, no detectable elution took place. There were similar changes with the other strains, although with the PR8 strain, the eluting ability was more heat-resistant.

TABLE II

Effect of Heat on Adsorption and Elution of Influenza Virus from Red Cells

Strain		Titered	Heated at 56°C. for						
			0 min.	5 min.	10 min.	20 min.	30 min.	40 min.	60 min.
Lee(B)	1	Preliminary titration on concentrated virus....	1024	1175	1270	1560	1910	1910	2048
	2	Before adsorption..	362	417	294	275	194	194	194
	3	Supernatant after adsorption	56	24	21	12	11	7	11
	4	Units of virus adsorbed .. .	306	393	273	263	183	187	183
	5	Eluted virus .	447	447	84	5	<2	<2	<2
	6	Adsorbed virus eluted, per cent.	100*	100*	31	1.9	<1.1	<1.1	<1.1
PR8(A)	1	Preliminary titration on concentrated virus	676	835	1024	1024	1175	1450	1450
	2	Before adsorption..	779	722	512	479	362	338	447
	3	Supernatant after adsorption .. .	45	24	13	10	10	5	7
	4	Units of virus adsorbed... . .	734	698	499	469	352	333	440
	5	Eluted virus. .	1175	79	49	28	39	23	23
	6	Adsorbed virus eluted, per cent..	100*	11	10	6	11	7	5

* Significantly more hemagglutinin was eluted than was adsorbed.

In Table II, a similar experiment is recorded in which the virus suspensions used were concentrated by centrifugation and in which the titers of the concentrates increased on heating (line 1), possibly due to disaggregation. An attempt was made to use the same number of agglutinating units in each sample, based on these figures (Table II, line 2) but, during the lapse of time (20 hours) between the preliminary titrations and the final test, the hemagglutinin levels in the specimens heated longest dropped from their calculated level by an amount which would indicate that the specimens had returned to their original (preheated) level in that time. Nevertheless, the experiment shows clearly the same phenomenon revealed in Table I. Again, the PR8

strain showed greater resistance than the Lee strain in the loss of eluting capacity[1] but in this experiment, the decrease in elution could not be ascribed to a lack of sufficient adsorbed hemagglutinin.

These data (Tables I and II) and their interpretation form the basis for understanding the further experiments in this paper on the agglutinin inhibitor of normal serum. In terms of the author's enzyme-substrate hypothesis of the action of influenza virus on red cells (4), virus which has been heated at 56°C. still retains enough of its configuration to combine specifically with the substrate (adsorption on red cells) but has lost its enzymatic capacity to split it (lack of elution). Where influenza virus is active in splitting the receptor substance, the union between virus and cell is temporary and rapidly changing while with heated virus, where no receptor destruction takes place, the union is much more firm and binding. The consequences of this hypothesis are interesting in relation to experiments with serum inhibitor.

Destruction of Serum Inhibitor by Influenza Virus as Measured by Heated (56°C.) Virus

In a previous paper a number of experiments were described (1) which pointed to a strong basic similarity between the virus receptor of red cells and the normal inhibitor which occurs in many sera. The similarity included the destruction of inhibitor in the presence of active influenza virus. Since unheated virus destroys both serum inhibitor and red cell receptors the demonstration of inhibition in the presence of active virus is most complicated, because after mixing virus and serum the virus is probably not firmly bound to the inhibitor which it is constantly inactivating, and hence may become available for adsorption to red cells during the incubation period of the test. This labile combination of inhibitor and virus may lower the inhibition values obtained. If virus heated at 56°C. has lost the power of splitting the inhibitor as well as the red cell receptor the union between them should be firm, possibly irreversible, as shown in the previous experiments with red cells, and one would expect higher inhibitory levels with heated preparations, as Francis observed. The use of heated virus should offer more consistent values for the measurement of the serum-inhibiting effect.

The Effect of Incubation Time on the Inhibition Obtained with Heated (56°C.) Virus.—Before beginning tests on the destruction of virus inhibitor in serum, a

[1] The phenomenon of an increase in hemagglutinin titer immediately following heating, followed by a return to the former level after a period of hours has been repeatedly observed. In Table II it can also be seen that in some instances the titer of the eluted virus was higher than that of the adsorbed virus. These shifts in titer have been seen only with virus concentrated by centrifugation, in which case there is undoubtedly aggregation of virus particles. This is indicated by the frequent loss of a large part of the total hemagglutinin activity on centrifugal concentration. Apparently heating temporarily disperses the particles and elution does so more permanently.

preliminary experiment was done on the effect on the inhibitory titer of prolonged incubation of heat-inactivated (56°C.) virus with serum.

For this experiment the L230(A) strain was used which is notable for its high inhibition levels obtained with normal serum. A number of sets of serial dilutions of normal rabbit serum

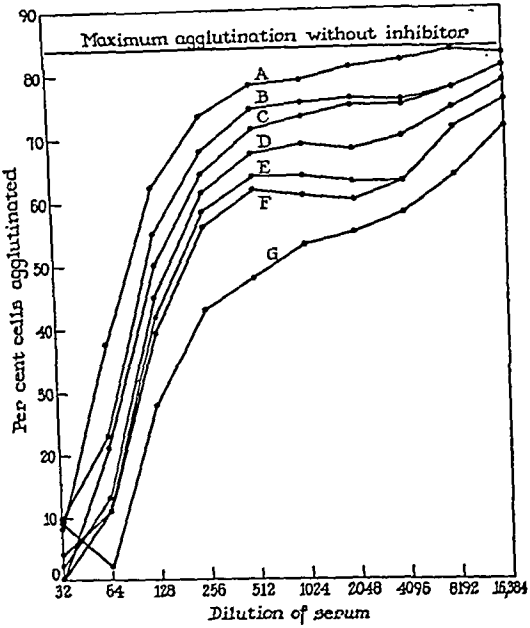

FIG. 1. Inhibition of hemagglutination by normal rabbit serum as tested with influenza virus, strain L230. The virus had been heated at 56°C. for 30 minutes and was used at a level of 12 units. Virus and serum dilutions were mixed and incubated at room temperature for various periods before the red cells were added. Line A, no incubation. Line B, 15 minutes incubation. Line C, 30 minutes. Line D, 1 hour. Line E, 2 hours. Line F, 3 hours. Line G, 20 hours incubation.

were made. Strain L230 (heat-inactivated 56°C.) was added to serum dilutions in a concentration such that the final hemagglutinin titer in each tube would be 12 units. The virus was added to sets of dilutions at appropriate intervals so that when cells were added to all the sets of dilutions the heated virus had incubated with the serum dilutions at room temperature for from zero minutes to 20 hours. Seventy-five minutes after adding the cells the tests were read. The degree of agglutination in each tube has been plotted in Fig. 1.

The results were striking and consistent throughout in showing that the virus which had been in contact with serum the least time before the addition of cells showed the least inhibition and that incubation of serum-virus mixtures resulted in progressively *increasing* inhibition up to the final 20 hour period. Whether equilibrium had been reached at 20 hours is not known. The increasing inhibition took place in all dilutions but was most marked at higher

dilutions. Virus control samples showed no significant drop in hemagglutinin titer when left at room temperature or in the ice box over the 20 hour period, and hence this factor probably does not enter into the explanation of the increase of inhibitory titer obtained. The increase in inhibition per unit time was most marked in the first 15 minutes and least in the final 17 hours, which is typical of adsorption curves in general.

This result obtained with heated virus is directly opposite to that previously obtained with incubation of active virus with serum (1), in that incubation with heated virus gave *increasing* inhibition while with unheated virus there was *decreasing* inhibition and this lends further support to the contention that with heated virus (56°C.) the ability to split the inhibitor is lost with retention of the capacity of the virus to combine with inhibitor.

The destruction of inhibitor in serum by active unheated virus could be further clarified if such treated sera could be tested with inactive virus. The problem in conducting such a test is to get rid of the unheated virus used for treatment so that it will not interfere with the heated virus of the final test. This may be done in two ways: (1) Treating serum with unheated virus and then heating the virus-serum mixtures after varying intervals at 56°C. in order to partially inactivate the virus and test the treated mixtures for inhibition with the virus heated in the presence of the serum. (2) Treating serum with unheated virus and heating after varying intervals at 65°C. in order to completely inactivate the virus present, and to conduct the inhibition tests with newly added virus which had been inactivated at 56°C. Both tests are useful and both have some disadvantages.

Measurement of Inhibitor Destruction in Serum by Titration with Virus Inactivated in the Presence of Serum.—

In this experiment a number of series of dilutions of normal rabbit serum were prepared. Active unheated Lee virus (18 units final concentration) was added at one time to a number of sets of dilutions, which after varying intervals were placed at 56°C. for 30 minutes. Before and after heating the mixtures were kept at room temperature; the longest period of incubation with unheated virus was 20 hours. Controls were tested in which previously heated virus (56°C.) was added to a set of serum dilutions 20.50 hours before cells were added (Fig. 2, line E) and in which preheated virus was added immediately before the addition of cells (Fig. 2, line D). Thus each set of dilutions, except line D, had had the same length of incubation at room temperature, the variables being the length of time before and after heating. Finally red cells were added to all the tubes and after 75 minutes the per cent of cells agglutinated was measured in every tube by a densitometer.

In such an experiment the question arises as to whether differences in inhibition might not, as in the previous experiment (Fig. 1), be due to differences in incubation time of serum with heated virus. Fortunately the Lee strain, used in this experiment does not show the large shifts in inhibition (lines D and E) with incubation which were found with strain L230. In addition, the

variation in incubation time in the experiments represented by lines B, C, and D was small (19.50, 20.25, and 20.50 hours respectively) and probably not significant in terms of equilibrium between heated virus and inhibitor. The drop in hemagglutinin titer of virus controls with heating at 56°C. was small

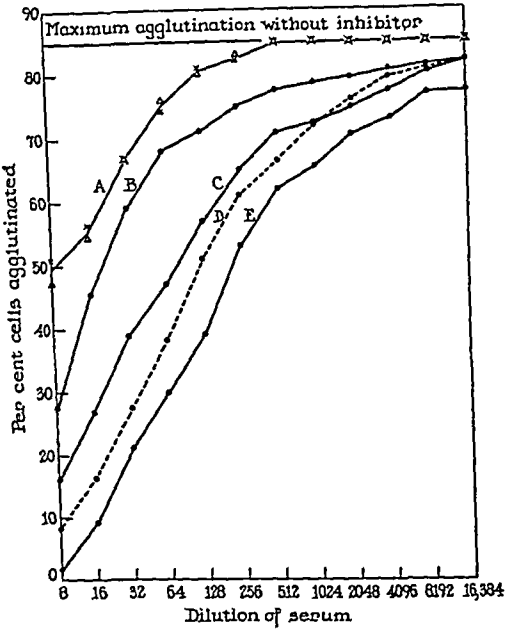

FIG. 2. Agglutination inhibition tests with normal rabbit serum, treated for various times with unheated Lee virus (18 units). The virus was added to the serum dilutions and inactivation was carried out at 56°C. for 30 minutes. The inhibition of the heat-inactivated virus was tested.

Line A (duplicate determinations), serum dilution treated for 20 hours with unheated virus and inactivated at 56°C. Line B, similar treatment in active state for 1 hour. Line C, treatment in active state for 15 minutes. Line D, preheated virus (56°C.) added to serum just before the test. Line E, preheated virus (56°C.) added to serum 20.5 hours before the test.

and uniform throughout the experiment. Though not indicated in the figure the inhibition effect was the same whether preheated virus was added to serum or the virus was heated immediately after addition to serum.

The exposure of normal rabbit serum to unheated virus resulted in a progressive and marked drop in the degree of inhibition obtained in a test with heat-inactivated virus. The decrease in inhibition was fairly uniform in all dilutions tested and whether lines D or E be taken as a base line for the inhibitory activity of untreated serum, the level dropped about 90 per cent in 3 hours and 95 per cent in 20 hours. While it is felt that this experiment fur-

nishes the most satisfactory evidence thus far for the inactivation of serum inhibitor by untreated virus, the method employed has the limitation that the shift in inhibition level must be measured by the same amount of the same strain which has been used for destroying the inhibitor. This objection is overcome in the second method.

Measurement of Destruction of Serum Inhibitor by Active Virus Which Is Destroyed by Heating at 65°C. before Testing with Inactivated (56°C.) Virus.— The advantages of destroying the active virus after it has acted on serum before

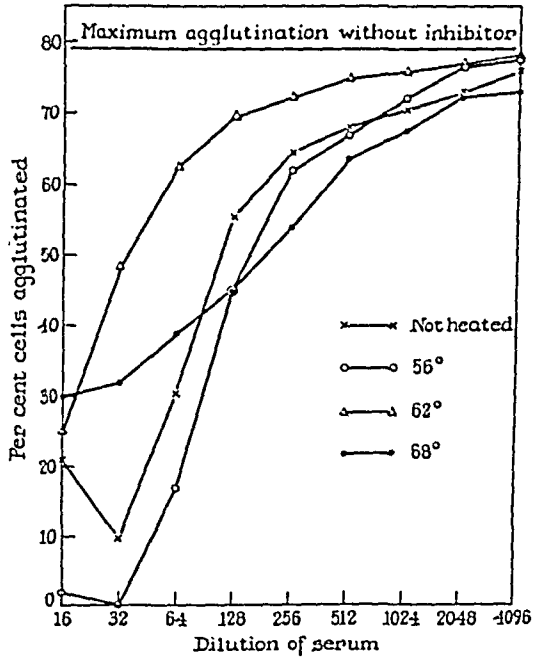

FIG. 3. Inhibition of agglutination of Lee virus by rabbit sera heated at several temperatures for 30 minutes. The sera were titered with 4.9 units of heated (56°C.) Lee virus.

testing with newly added virus are obvious since it multiplies the number of ways in which inactivated serum can be tested. This technique was not attractive at first because of the peculiar behavior of the inhibitor when serum is heated at temperatures above 60°C. McCrea has stated that the inhibitor of influenza virus hemagglutination is destroyed by heating for 15 to 20 minutes at 62°C. (5). We have not been able to confirm this statement and the behavior of inhibitor on heating is complex as will be shown below.

In a preliminary experiment, normal rabbit serum unheated, and heated at 56°, 62°, and 68°C. for 30 minutes was tested in inhibition tests with 5 units of heated (56°C.) Lee virus. The test (Fig. 3) shows no significant difference in inhibition curves between untreated serum and serum heated at 56°C. Serum heated at 62°C. showed a drop in inhibitory titer of about fourfold,

while serum heated at an even higher temperature (68°C.) showed a consistent and marked increase in inhibition over the level of the 62°C.) serum. At higher dilutions the serum heated at the highest temperature showed as much and possibly somewhat more inhibition than unheated serum but the shape of the curve is peculiar and the inhibition in lower dilutions is markedly less than that of normal serum. This is tentatively interpreted as interference with the inhibitor by various serum constituents, the interfering agents varying in activity with changes on heating, and their effect is diluted out more rapidly than the inhibition effect. Further evidence along this line will be offered in another paper.

In spite of the complicated behavior of inhibitor in serum heated at temperatures above 60°C. an attempt was made to use heat for inactivating virus in the presence of serum. Preliminary tests in the hemagglutinin titer variation with temperature showed a wide difference in susceptibility of various strains, and nothing less than 40 minutes at 65°C. would surely inactivate the hemagglutin of all preparations.

In this experiment two virus strains were used, Lee(B) and L230(A). Both were added in unheated state to normal rabbit serum. The final dilution of serum was 1:4 and the final titer of hemagglutinin with each strain was 32. These mixtures were allowed to stand at room temperature for 20 hours. Samples were removed periodically and heated at 65°C. for 40 minutes. The same procedure was carried out with serum and heat-inactivated preparations (56°C.). In addition, controls of serum (without virus added) heated at 65°C. and at 56°C. were tested. All specimens were titered for inhibition with 3 units of heat-inactivated virus (56°C.) of the two strains used. The results are shown in Figures 4, 5, 6, and 7.

In Fig. 4 the results of treating serum with heated or unheated Lee virus and testing with heated (56°C.) Lee virus are shown. Lines D and E show the effects of heating serum alone at 65°C., the titer being reduced about twofold over the 56°C. control. Curves A, B, and C show that the effect of incubating serum with unheated Lee virus was a marked reduction in the inhibitory level, similar to that seen in Fig. 2. Since these curves are not parallel, but converge in the higher serum dilutions, it is not clear how much inhibitor was destroyed. At the 60 per cent agglutination level the destruction would appear to be only 30 per cent in 1 hour while at the 10 per cent level it is close to 90 per cent. This convergence of curves cannot be explained satisfactorily at present but may be due to multiple factors affecting the inhibition as suggested above. When the same sera were tested with a second virus (L230) the convergence was not marked.

When virus (Lee) which had been heated at 56°C. was added to normal rabbit serum and inactivated at 65°C. after varying intervals up to 20 hours there was no change in inhibitory level and the curve at 20 hours (Fig. 4, line D, triangles) coincided with that of untreated serum heated at 65°C. (line D, ×), thus confirming the fact that partially heat-inactivated virus (56°C.) has lost

its capacity to destroy inhibitor. A further control of active virus added to serum and inactivated at 65°C. at once gave values coincident with line D.

Fig. 5 shows the results of testing the same serum samples of the previous experiment (lines A, B, C, and D in Fig. 4) with another virus, strain L230A). The inhibition curves with this strain have a characteristic appearance with a

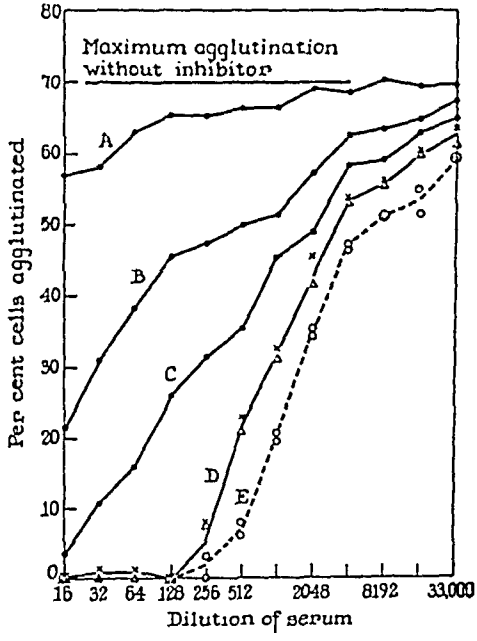

FIG. 4. Agglutination inhibition tests with normal rabbit serum treated for various periods with unheated Lee virus. The virus was destroyed by heating the serum-virus mixtures at 65°C. for 40 minutes and the sera were tested for inhibitory titer against 3 units of heated (56°C.) Lee virus. This amount of Lee virus was sufficient to agglutinate 70 per cent of the red cells in the absence of any inhibitor.

Line A, serum treated with unheated Lee virus for 20 hours. Line B, treated for 3 hours. Line C, for 1 hour. Line D (×), for zero hours. Line D (triangles), serum treated with heated (56°C.) Lee virus for 20 hours. Line E (open circles, duplicate determinations), control serum, no virus added and heating to 56°C. only.

plateau in the center and as used with these sera are consistent with the maximum inhibitor destruction found with the homologous (Lee) strain. The curves are much more nearly parallel than with the Lee strain and show 60 to 75 per cent destruction of inhibitor in 1 hour, over 90 per cent in 3 hours, and more than 99 per cent in 20 hours

In Fig. 6 are shown the results of treating the same normal rabbit serum with strain L230 at the same strength in terms of agglutinating units as the Lee strain in the experiment above. This is a newly isolated strain which shows very high inhibition levels with normal serum in the unheated state, much

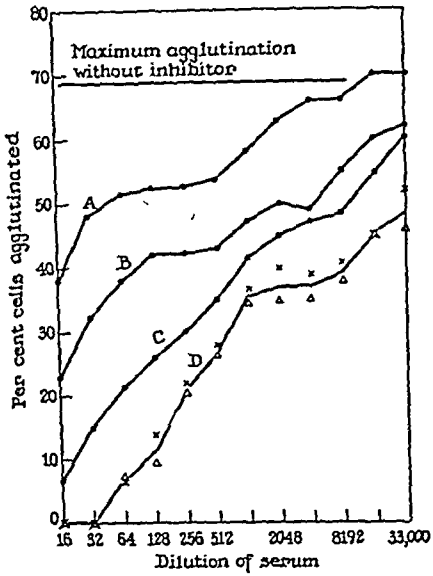

FIG. 5. The sera are the same ones tested in Fig. 4. In this experiment they were tested for inhibitory titer with strain L230 heat-inactivated at 56°C. and used at 3 units.

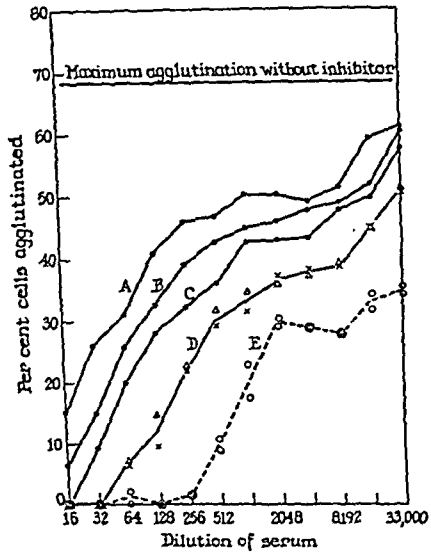

FIG. 6. Agglutination inhibition tests with normal rabbit serum treated for various periods with unheated L230(A) influenza virus. The virus was destroyed by heating at 65°C. for 30 minutes and the sera were tested for inhibitory titer against strain L230 heated (56°C.). Line A, exposure of serum to unheated virus for 20 hours. Line B, for 3 hours. Line C, for 1 hour. Line D (×), for zero hours. Line D (triangles), exposure to heated (56°C.) L230 virus for 20 hours. Line E (open circles, duplicate determinations) control serum, no virus added and heating to 56°C. only.

higher than Lee or PR8. One might deduce from this that this strain splits the inhibitor at a lower rate than the Lee strain. However, its elution from red cells is almost as rapid as that of the Lee strain and the difference in rate of destruction shown in Fig. 6 is only slightly less than the rate with Lee, almost 75 per cent in 1 hour and over 90 per cent in 20 hours. Again, as in Fig. 5, the curves have a characteristic plateau; within the limits of experimental error they are parallel and serum treatment with heated L230 virus (56°C.) had no effect on the inhibitory titer. Heating normal untreated serum at 65°C.

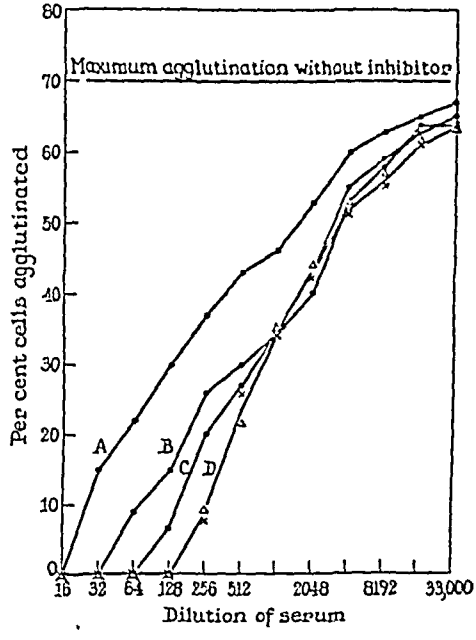

FIG. 7. The sera are the same as those tested in Fig. 6. In this experiment the sera were exposed to unheated virus of strain L230 but were tested for inhibitory titer with heated (56°C.) strain Lee, 3 units.

lowered the inhibitory titer by 75 per cent for this strain as opposed to 50 per cent for the Lee strain which is a significant difference. The same L230-treated sera when tested with the Lee strain gave the picture seen in Fig. 7. In the region of 10 to 20 per cent agglutination the reduction of inhibitor was consistent with the amount of inhibitor destruction seen in Fig. 6, but the curves converged very markedly in the zones of lesser inhibition. Again it seems likely that this lack of parallelism may be due to interfering serum factors, or possibly, multiple inhibiting factors, one of which affects the Lee strain at high dilution more than it affects agglutination by the L230 strain.

DISCUSSION

The removal of the destructive or enzymatic capacity of a virus without completely destroying its capacity to combine with red cell receptors or serum

inhibitor provides a unique tool for the quantitation of the inhibitory action of serum on virus hemagglutination. By means of inhibition titrations with active virus it has been possible to demonstrate more conclusively than before that unheated virus has the ability to rapidly destroy the virus inhibitor in serum. While it may be questioned at this time whether it is the virus or something else in virus suspensions which destroys the serum inhibitor the bulk of evidence at the moment is in favor of the association of the activity with the virus particle. Virus partially purified (by centrifugation or by adsorption on an elution from red cells) also possesses the inhibitor-destroying capacity, while very low titer supernatant fluids from which the virus has been removed by centrifugation do not. A more precise proof that the activity is associated with the virus itself may be possible after purification of the serum inhibitor.

The use of heat-inactivated influenza virus for measuring serum inhibitor provides a rather accurate means of titrating this factor and one which is also very sensitive. In tests in which the L230 strain was used and-left in contact with the serum dilutions for several hours a significant degree of inhibition of agglutination has been detected in sera diluted over 100,000 times. Assuming that rabbit serum contains a 1 per cent concentration of this substance, the limit of detection would be of the order of part 1 in 10 million.

The use of inhibition curves in this paper might have been avoided by the use of simple end points (*e.g.* the dilution of serum at which the agglutination of red cells is 50 per cent complete), in which case the results obtained would have been qualitatively similar to the ones presented. However, the entire curves tell a more complete story and this is especially important where the inhibitor is being measured in whole serum and heated serum, because of the complicating factors which interfere with the inhibition.

Of the two methods of measuring inhibitor destruction by unheated virus the second is more versatile. When testing for inhibitor in whole serum the marked degree of loss in inhibitor on heating at 65°C. is disturbing. As will be shown in another paper, this objection does not enter into the titration of purified inhibitor.

Burnet and his colleagues (6, 7) have published two preliminary notes in which they refer to a method of measuring serum inhibitor with heat-inactivated virus but at the time of the present report a detailed description of their method was not available to the author.

SUMMARY

Evidence has been offered that influenza virus which has been heated at 56°C. for 30 or more minutes loses some of its capacity to agglutinate red cells and may completely lose its power to elute from cells on which it has been adsorbed. Such heat-inactivated virus does not possess the capacity to

destroy the virus inhibitor in normal rabbit serum and this appears to be the explanation of the higher agglutinin inhibitory levels obtained with serum and heated virus as compared with serum and untreated virus.

The heat-inactivated virus can be used to measure the inhibitor substance in normal rabbit serum. By two different methods it has been demonstrated that the inhibitor is destroyed in the presence of unheated influenza virus, as measured by inhibition titrations with virus inactivated at 56°C. The destruction of inhibitor by virus of either type A or B can be measured by virus of either type with similar results.

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THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON BLOOD PRESSURE, RENAL FUNCTION, AND ELECTROLYTE PATTERN IN THE INTACT RAT*

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The problem of the relation between experimental renal hypertension and essential hypertension in man, hinging on the primacy of the kidney, has assumed a critical importance in recent years. The alternate positions have been summarized by Smith, Goldring, and Chasis (1), Goldring and Chasis (2), and more recently by Goldblatt (3). Recent work by Selye and his colleagues implicating the adrenal cortex in the etiology of the hypertensive syndrome has, in a sense, offered an intermediate position (4, 5). These workers demonstrated, first in the chick (6), and later, in the rat, dog, and monkey (7), that overdosage with desoxycorticosterone acetate (DCA) could produce hypertension and renal damage in animals sensitized with saline. Later these workers demonstrated that an endogenous overproduction of cortical hormones resulting from stress could also cause this nephrosclerosis and hypertension in the rat (8). The rôle of the adrenal in experimental hypertension in animals has been confirmed by many workers, more recently by Anderson, Page, Li, and Ogden (9), and Page, Ogden, and Anderson (10), who showed that renal hypertension could be restored in hypophysectomized rats by administration of the adrenocorticotropic hormone alone, and to a lesser extent by desoxycorticosterone or adrenal cortical extract.

Clinical support for Selye's findings has come from several sources. Perera and his coworkers have demonstrated that patients with Addison's disease being treated with DCA may develop elevated blood pressure (11–13), while F. Selye has shown the presence of an elevated Na/Cl ratio—supposedly characteristic of DCA overdosage (5)—in some patients with hypertension (14). More recently Hoagland (15) has demonstrated strikingly the intimate correlation between cortical hormone production and stress in man.

The idea that the adrenal plays a primary rôle in the development of hypertension has, however, not gone uncriticized (3, 16). Thus Goldblatt (3) has stated, "it is doubtful that any of the known endocrines plays a primary part in either essential hypertension associated with vascular disease in man, or in experimental renal hypertension in animals." Further it must be admitted that even though the dosages of DCA usually employed are large, saline feeding, preferably coupled with uninephrectomy or other intensifying measures, seems necessary to elicit the full character of the lesions (17).

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It seemed to us that insufficient evidence had been presented either for discounting or affirming the rôle of the adrenal cortex in the genesis of hypertension. From the literature, it seemed possible that reasons for disagreement among authors might rest in part with the mode of administration and the quantity of DCA used as well as with the technique of blood pressure determinations. With this in mind, several preliminary experiments were carried out in which we were able to determine that small doses of DCA administered in pellet form were capable of raising blood pressure and causing kidney damage in the intact animal. Encapsulation of the pellets occurred within about 14 days and was followed by the disappearance of the effects. If, instead of a single large pellet, small pellets were implanted singly every 10 days into a new site, progressive changes were consistently observed. Pellets are recovered amazingly intact, indicating slow rates of absorption. In these experiments it was also noted that sustained increases of blood pressure were paralleled by increases in heart weight due largely to ventricular hypertrophy determined histologically. It became evident that the determination of heart weight was an objective means of confirming the presence of chronic hypertension in experiments involving treatment with DCA.

With this background, the progressive changes in renal function, electrolyte pattern, and blood pressure following the administration of DCA to intact rats were investigated.

EXPERIMENTAL

Four groups of twenty male albino rats were maintained for 42 days. The animals were 28 days old and approximately evenly matched in weight at the start. They were housed five to a cage and received Purina Fox Chow *ad libitum*. The first and third groups drank tap water, while the second and fourth received 1 per cent saline. In addition, the third and fourth groups carried subcutaneous implants of DCA throughout the experiment. There were thus: group 1, intact control; group 2, intact control drinking saline; group 3, animals with DCA pellet; and group 4, animals with DCA pellet also drinking saline.

As already mentioned, lead experiments had demonstrated that for this type of work the mode of administration of DCA is important. In this experiment, 75 mg. pellets of DCA[1] were broken into three roughly equal parts, and each third was separately implanted on the 1st, 11th, and 25th days of the experiment. On the 42nd day the animals were sacrificed. Tissues were fixed for subsequent histological examination, and the kidneys and heart were weighed after 6 hours' fixation.

During the course of the experiment blood electrolyte studies (18) were carried out on individual animals once every 2 weeks beginning on the 12th day. Similarly, clearance studies using inulin and sodium *p*-aminohippurate were performed every 2 weeks beginning on the 10th day. Blood for both procedures was obtained by heart puncture. Half of each group of twenty animals were thus used throughout for electrolyte studies, while the other half were used for renal function tests, so that no animal was subjected to heart puncture oftener than once in 14 days. This is an ample recovery period as shown by the rapid return of normal behaviour in the animals, as well as by hematocrit studies.

[1] Schering cortate.

Briefly the clearance technique is as follows: PAH solution (12.5 mg./cc. in 2 per cent sodium sulfate) is injected into the lumbar region in accordance with body weight. The total dose in normal animals weighing 110 gm. is 40 mg., for animals weighing 150 gm., 47 mg., and for animals weighing 200 gm., 55 mg., with intermediate doses for intermediate weights. Where renal function is impaired, smaller doses may suffice to yield the correct plasma level of 5 to 7 mg per cent at 50 minutes.

Immediately following this injection, 3 cc. of warm 2 per cent inulin solution is injected intraperitoneally. The completion of this second injection marks the start of the urine collection period, and the rat is immediately placed into a metabolism funnel.

Fifty minutes after injection, the rat is picked up over the funnel and the bladder is drained by suprapubic pressure, although micturition is usually quite free and spontaneous. Immediately following urine collection, 0.75 cc. of blood is obtained by heart puncture in the 51st minute, using a 24 or 25 gauge heparinized needle. The animal is returned to its cage unharmed and the blood and urine analysed for inulin and PAH.

The rationale for this procedure and the rigid criteria necessary for its execution and for calculation of valid clearance data have already been discussed in detail elsewhere (19). Thirty-two animals are handled per day.

Blood pressure determinations were carried out on all animals on the day before the electrolyte or clearance test, using the method of Byrom and Wilson (20) with ether anesthetic. While this method may fail to record a raised pressure in the occasional animal which is actually hypertensive, it has been our experience that it never falsely indicates a rise.

Table I presents the data for the 2nd week of the experiment.

Blood Pressure.—Blood pressure was significantly elevated in both groups 3 and 4; *i.e.*, DCA and DCA-saline. Four animals in group 3 and six animals in group 4 had pressures exceeding the control average by more than twice the standard deviation of the latter value.

Renal Function.—Group 2, receiving saline, but no DCA, showed an increase in glomerular filtration rate (GFR, C_{IN}) and in filtration fraction (FF). On the other hand, renal function appeared undisturbed in the two DCA groups. When group 4 is compared with its saline-fed control, however, the administration of DCA is seen to have negated the expected rise in GFR and FF. This difference is statistically significant.

Plasma Electrolytes.—The only significant electrolyte change at this period is the fall in plasma K observed in groups 3 and 4; *i. e.*, DCA and DCA-saline. Other trends, however, are interesting in the light of later observations. Thus the slight elevation in both Na and Cl seen in group 2 was maintained throughout the later experimental period. The small rise in Na occurring in both groups receiving DCA compared to their controls was increased later. At this time an increase in the Na/Cl ratio had occurred only in the DCA-saline group compared with its control, but this change became increasingly significant. The total ionic concentration indicated by Na plus K remains normal, the fall in K being insufficient to alter this total.

Table I presents the data obtained in the 4th week of the experiment.

Blood Pressure.—Blood pressure was significantly elevated in the two DCA groups and had progressed beyond that previously observed. Six animals in group 3 and eight animals in group 4 had pressures exceeding the control average by more than twice the standard deviation of the latter value.

Renal Function.—Renal function was definitely altered at this time. The aberrations observed at 2 weeks in the saline-fed group disappeared although the slight hyperemia indicated in the first test (C_{PAH}/Tm_{PAH}, renal plasma flow per unit of excretory tissue), still appears to

TABLE I

	2nd week				4th week				6th week				
Group No.	1	2	3	4	1	2	3	4	1	2	3	4	
Treatment	Control	Saline	DCA	DCA-saline	Control	Saline	DCA	DCA-saline	Control	Saline	DCA	DCA-saline	No. of animals per group
Blood pressure	101 ±11.7	97 ±9.5	110 ±13.9	113 ±13.3	100 ±13.8	96 ±13.0	115 ±13.0	120 ±13.6	108 ±14.8	103 ±10.4	117 ±18.8	129 ±19.6	16
C_{IN}, *cc./100 cm.²*	0.34 ±0.07	0.48 ±0.05	0.34 ±0.04	0.40 ±0.09	0.34 ±0.07	0.34 ±0.07	0.34 ±0.06	0.31 ±0.09	0.35 ±0.03	0.30 ±0.04	0.32 ±0.07	0.22 ±0.03	8
C_{PAH}, *cc./100 cm.²*	2.18 ±0.26	2.34 ±0.35	2.44 ±0.61	2.48 ±0.45	2.55 ±0.36	2.57 ±0.44	2.16 ±0.41	1.76 ±0.30	2.47 ±0.52	2.76 ±0.46	2.37 ±0.36	1.55 ±0.44	8
T_{mPAH}, *mg./100 cm.²*	0.119 ±0.012	0.118 ±0.014	0.128 ±0.014	0.126 ±0.013	0.126 ±0.012	0.115 ±0.012	0.111 ±0.010	0.107 ±0.007	0.118 ±0.020	0.125 ±0.014	0.107 ±0.013	0.093 ±0.012	8
FF as per cent	14.5 ±4.6	20.7 ±3.6	14.7 ±2.4	15.3 ±5.0	13.3 ±2.0	13.8 ±1.9	16.4 ±2.0	17.6 ±3.6	16.2 ±4.0	11.2 ±2.1	13.5 ±3.6	15.6 ±6.0	8
C_{PAH}/T_{mPAH}	18.6 ±2.3	20.0 ±2.6	19.8 ±5.1	19.6 ±3.3	20.3 ±2.7	22.4 ±2.9	18.7 ±2.4	17.7 ±3.2	20.9 ±1.4	20.8 ±2.5	22.2 ±3.0	16.7 ±2.8	8
Na, *m. eq.*	149.2 ±2.7	152.8 ±2.4	151.8 ±2.0	153.8 ±3.3	151.4 ±4.8	152.5 ±6.6	155.7 ±6.6	155.1 ±3.1	148.0 ±1.3	149.2 ±2.0	151.7 ±4.0	158.6 ±3.7	8
Cl, *m. eq.*	98.1 ±1.5	103.9 ±0.6	99.1 ±3.9	100.2 ±2.0	103.6 ±1.6	104.8 ±2.1	103.6 ±3.3	94.6 ±2.0	104.8 ±1.3	106.3 ±1.0	98.1 ±1.4	95.7 ±1.1	8
K, *m. eq.*	8.0 ±0.7	5.0 ±0.3	4.2 ±0.2	3.8 ±0.3	6.8 ±0.5	6.2 ±0.6	3.5 ±0.4	3.2 ±0.6	5.4 ±0.8	5.3 ±0.4	4.1 ±0.6	2.7 ±0.6	8
Ca, *m. eq.*	—	—	—	—	6.9 ±0.1	6.8 ±0.3	6.5 ±0.2	6.7 ±0.2	6.4 ±0.1	6.3 ±0.3	6.3 ±0.3	6.0 ±0.4	8
Na/Cl	1.52	1.47	1.53	1.53	1.46	1.45	1.50	1.64	1.41	1.40	1.57	1.65	8

Na + K.	157.2	157.8	156.0	157.6	158.2	158.7	159.2	158.3	153.4	154.5	159.8	161.3	8
Hematocrit, *per cent*									48 ±1	47 ±2	47 ±1	48 ±3	6
Heart rate, *per min*.....									390 ±50	420 ±40	380 ±40	360 ±50	16
Heart weight, *mg./100 cm.*^{3}.....									188 ±12	191 ±11	209 ±24	228 ±22	16
Kidney weight, *mg./100 cm.*^{3}.....									465 ±54	474 ±30	544 ±67	683 ±58	16
Final body weight, *gm.*									193 ±21	201 ±22	204 ±24	158 ±24	16

The inulin clearance, C_{IN} measures the glomerular filtration rate (GFR). The clearance of PAH, C_{PAH}, measures the renal plasma flow (RPF) at the plasma levels of PAH used here. T_{mPAH} represents the minute tubular excretion of PAH (total excretion of PAH less the amount filtered) and hence measures the functioning tubular excretory mass. The ratio C_{IN}/C_{PAH} represents the fraction of plasma filtered at the glomerulus and is termed filtration fraction (FF). The ratio C_{PAH}/T_{mPAH} expresses the plasma flow for each unit of functioning tubular excretory tissue.

be present. Contrasting the DCA groups with their respective controls, it is readily seen that GFR was maintained but the total renal plasma flow, (RPF, C_{PAH}), was reduced. Similarly, the mass of functioning tubular excretory tissue, (Tm_{PAH}), was reduced in these groups. Reflecting these changes was a rise in FF which probably indicates constriction of the efferent arteriole since there was also a relative ischemia of kidney tubules as shown by the decline in C_{PAH}/Tm_{PAH}, particularly in group 4.

Plasma Electrolytes.—Clear cut changes in electrolyte pattern were also well established at this time. The elevation in both Na and Cl in the saline-fed group 2 was maintained. Plasma Na was significantly increased in both groups receiving DCA, a change previously indicated, and the Na/Cl ratio was likewise significantly elevated in these groups. The increase in this ratio was aggravated in the DCA-saline group by the definite fall in plasma Cl, despite the fact that this group received extra Cl from the NaCl. The fall in plasma K in both DCA groups was still pronounced but had not progressed beyond the level previously observed. The total ionic concentration indicated by Na plus K remained normal, the fall in K being balanced by the rise in Na. Plasma calcium was normal in all groups.

Table I presents the data obtained in the 6th week of the experiment.

Blood Pressure.—Blood pressure was significantly elevated in the DCA-saline group and higher than previously observed. On the other hand, while the pressure in the DCA group was still elevated, it had not progressed and indeed, it is questionable whether any significant elevation actually existed at this time. (The advance of the average blood pressure value in the control animals during the 6 week period is our usual finding for growing animals.) Four animals in group 3 and ten animals in group 4 had pressures exceeding the control average by more than twice the standard deviation of the latter value.

Renal Function.—The changes in renal function at this time are interesting. The temporary aberrations in the saline-fed group 2 have disappeared and the FF is now rather reduced.

In the DCA-treated animals of group 3 some adaptation must have been effected, for renal function was now well within normal limits, except that Tm still appeared reduced. These findings are in obvious contrast to the observations of the previous period, and suggest that the kidney changes may be to some extent reversible.

In the DCA-saline group the changes observed at the end of the 4th week had intensified. The GFR was no longer maintained, indicating a considerable degree of interference with the filtering mechanism. Renal plasma flow was cut to only a little more than half the normal value, and the functioning excretory tubular mass was considerably reduced. These changes are even more striking in view of the considerable renal enlargement which occurred during the experiment. The FF was unchanged on the average but the broad spread of data indicates that as glomerular damage progressed the raised FF previously observed changed to a reduced FF with individual animals in various phases of the process. Renal ischemia was definitely present.

Plasma Electrolytes.—The electrolyte pattern differed from that previously observed only in degree. Thus in the saline-fed group 2 there was still the slight expected increase in both Na and Cl. In the two DCA groups the elevation of plasma Na was now more marked, while the depression of plasma Cl seen only in group 4, DCA-saline, at 4 weeks was now seen in both groups. The decline in plasma K was still present but in no greater degree. The elevation in the Na/Cl ratio was now more marked while the sum of Na plus K was also increased, the decreased K no longer being able to compensate for the increased Na. Plasma calcium remained undisturbed.

Organ Weights.—The hypertension observed during the course of the experiment was substantiated by the significant increase in heart weight which occurred in both DCA groups.

The increase in heart weight in the DCA group was less than that observed in the DCA-saline group paralleling the relative blood pressure increases noted. The kidney weights are interesting, for the significant renal enlargement observed in both DCA-treated groups indicates how poorly renal efficiency was actually maintained. It is similarly clear from the hematocrit values that the animals had adjusted to the previous withdrawals of blood.

Heart rate was determined under light nembutal anesthesia, a procedure which gives rates somewhat above the basal level (21). The absence of any significant change in heart rate makes it unlikely that the increased blood pressure was due to increased cardiac output.

Histological examination of the kidneys showed the presence of diffusely distributed early glomerular sclerosis in both DCA groups. No tubular damage or vascular lesions were seen and the glomerular lesions were actually minimal.

DISCUSSION

As a result of DCA treatment, renal structure and function were affected, the electrolyte pattern was upset, and the blood pressure rose. Each of these factors may be examined separately.

The Effect on the Kidney.—It is apparent that DCA alone in small doses is capable of interfering with renal function, while the addition of saline intensifies the process.

The first change is a decrease in renal plasma flow with the maintenance of normal filtration, findings suggesting efferent vascular constriction. At this time the flow of blood to each unit of functioning excretory tissue is normal so that no true ischemia is present. Later the mass of functioning tubular excretory tissue decreases while renal plasma flow decreases even more, so that renal ischemia occurs. In this later stage gross interference with filtration is present.

The pattern of renal functional change seems remarkably similar to that observed in essential hypertension in man. When the DCA administration was accompanied by saline feeding the process was progressive, while in those animals receiving DCA alone the vascular spasm and renal ischemia disappeared, suggesting the reversibility of the process, a finding reminiscent again of essential hypertension in man (22). Interestingly, the elevation of blood pressure in these latter animals became less apparent concurrently with the improvement in renal function.

Superficially the blood pressure elevation occurred well before the onset of renal interference, but it is obviously not possible to come to any definite conclusion as to the primacy of either the blood pressure increase or the renal damage from these data. Certainly, however, the rise in blood pressure may occur without evidence of renal ischemia.

The Effect on Electrolytes.—A progressive increase in plasma sodium occurs, together with a decrease in plasma chloride and potassium. These changes are in accord with reported observations following DCA administration both in animals and man. Selye, Hall, and Rowley (5) noted the elevation in Na/Cl ratio and the fall in potassium in rats receiving DCA with saline, but failed to

observe an actual rise in sodium with their methods. Similar findings were reported by F. Selye in some hypertensive patients (14). Knowlton, Loeb, Stoerk, and Seegal (17) found the same changes as here reported in rats, but did not mention the total increase in cations denoted by Na plus K, although it is indicated in their data. Similar results have been published by Ferrebee *et al.* (23). Thus, DCA alone in small doses is capable of interfering with the electrolyte balance, while the addition of saline intensifies the process. It seems to us of importance to establish whether or not the increase in the total of sodium and potassium represents an osmotic plasma to tissue differential which could be etiologically significant. The data seem to indicate that DCA causes sodium retention, that potassium is lost in a failing effort to maintain a normal total concentration of cations, and that chloride is lost passively, coupled with potassium. Further work is necessary on these points.

The Rise in Blood Pressure.—There does not seem to be any reasonable doubt that the blood pressure rose early in the experiment. It might be argued from a perusal of the absolute data that these elevations cannot be considered true hypertension. We would agree that an average blood pressure rise of 10 or 15 mm. Hg does not seem very much when compared with the magnitude of the figures usually associated with clinical and experimental renal hypertension. The actual figures here presented are, however, not absolute. The instrument, in our hands, records a pressure which probably is somewhat below the systolic level and the actual values obtained lie in a restricted range. Further, as pointed out earlier, in dealing with a process in which different animals are necessarily in different stages of the pathological disturbance, the average pressure rise is at most an arithmetic mean between hypertensive and normotensive animals. It is for this reason that in the description of results, the number of animals with blood pressure exceeding the average control level by more than twice its standard deviation is presented. Proof of the validity of the estimates rests on the demonstration of cardiac hypertrophy in the hypertensive animals.

Several workers have doubted that any of the known endocrine organs play a primary part in either essential hypertension in man or in experimental hypertension (3, 16). In the light of our present findings this thesis may be untenable. It has been demonstrated that a syndrome presenting certain marked similarities to essential hypertension can be produced in the intact rat by DCA. Further, it is clear that small amounts are sufficient to elicit the aberrations reported without resort to any intensifying measures, such as adding saline or reducing renal reserve by uninephrectomy. When these facts are added to the demonstrated responsiveness of the human adrenal cortex to stress, to recent reports on the blood pressure elevation observed in patients receiving DCA implants for Addison's disease, and when the renal functional alterations here reported are compared with those observed in man, it would appear that the rôle of the adrenal cortex cannot be lightly dismissed.

This is not to suggest that hypertension is an endocrine disease, but rather to indicate that there may be patients with hypertension of hormonal etiology to be distinguished from those in whom the disease is truly "essential."

SUMMARY

Small doses of DCA administered at intervals in pellet form are capable of raising the blood pressure, altering renal function, and changing the electrolyte pattern in the intact rat. The concomitant feeding of 1 per cent saline intensifies the process.

The elevation in blood pressure occurs prior to demonstrable changes in renal excretory function.

The alteration in renal function consists first of a reduction in C_{PAH} with the maintenance of a normal filtration rate. Filtration fraction is elevated while there is no reduction in renal plasma flow per unit of tubular excretory tissue. Later, filtration is interfered with and renal ischemia occurs.

The electrolyte change is characterized by a sustained fall in plasma K and Cl, a rise in plasma Na, an increase in the Na/Cl ratio, and finally an elevation of Na plus K. Plasma Ca is unaffected.

These observations suggest the possible etiological significance of the adrenal cortex in some types of hypertension.

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STUDIES ON SCRUB TYPHUS (TSUTSUGAMUSHI DISEASE)

III. Heterogenicity of Strains of R. tsutsugamushi as Demonstrated by Cross-Vaccination Studies*

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Although animals which recover from disease induced by one strain of *Rickettsia tsutsugamushi* are resistant to infection with heterologous strains (1–10), all organisms now grouped in this subgenus are not antigenically homogeneous. Evidence for heterogeneity is obtained in serological studies which employ complement fixation (11, 12), serum protection (13), and toxin-antitoxin techniques (14). Non-infectious vaccines of several types are capable of immunizing mice against the homologous strain of *R. tsutsugamushi* (15–17) but the degree of protection elicited by such materials against heterologous strains is unknown. The present report supplies information on this point and provides additional evidence of antigenic differences among the members of the scrub typhus group of agents.

Materials and Methods

Strains of R. tsutsugamushi.—The eight strains of *R. tsutsugamushi* employed in the current investigation were selected to represent the agent as obtained from various geographical areas and from different zoological sources; *i.e.*, men, mites, and rats. The origin and lineal descent of these strains are given in some detail in the following paragraphs since it appears desirable to establish these points clearly. Certain of the immunological and serological data which indicate the known relationships of the eight to each other, or to additional strains of *R. tsutsugamushi*, are summarized in Table I; this information has been presented in detail elsewhere (18). The toxin-antitoxin reaction is not mentioned in Table I because only the Gilliam strain has yielded a toxin and none of the antisera against the reference strains contains appreciable amounts of specific antitoxin (14).

The Imphal 8 strain was recovered late in 1943 from a pool of blood clots obtained from five patients with tsutsugamushi disease who were hospitalized in the Imphal Valley on the Indian-Burmese border. The characteristics of this strain shortly after isolation have been reported (19). In the present work, seed inoculum for the preparation of vaccine and material for challenging vaccinated mice were obtained from yolk sacs of the 47th to 53rd passages. Since the original isolation from human beings, this strain had been through one guinea pig, eight rabbits, one mouse, and the designated number of egg passages.

The Karp strain was originally obtained by Dr. E. Derrick of Brisbane, Australia, from

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blood taken in January, 1943, from a soldier who contracted the disease in the Buna-Guna area of New Guinea. The Karp agent was used previously in this laboratory for the prepara-

TABLE I

Antigenic Relationships of Strains of R. tsutsugamushi

Reference strains	Immune animals resist challenge with (strain)		Serological tests with antisera prepared against reference strains					
			Serum neutralization			Complement fixation		
			Challenge strain		Result*	Antigen		Result*
Imphal 8	Calcutta	(10‡)				Calcutta	(19)	++
Karp	Gilliam	(8)						
	Seerangayee	(8)						
	Buie (case 9)	(8)				Gilliam	(11)	+−
	Ceylon	(9)				Seerangayee	(11)	+−
	Calcutta	(9)				Buie (case 9)	(11)	+
	Imphal 8 or 13	(9)						
Kostival	Mite 21	(6)	Mite 21	(13)	+−			
	Mite 22	(6)	Seerangayee	(13)	+−			
Mite 21	Kostival	(6)	Kostival	(13)	+−			
			Seerangayee	(13)	+−			
Wild rat 235	135 Wild rat	(7)						
	370 Mite	(7)						
	Shope	(7)						
Pescadores	Niigata	(3)						
Seerangayee	6 Malayan strains	(2)						
	2 Sumatran strains	(2)	Kostival	(13)	+−	Gilliam	(11)	+−
	Karp	(8)	Mite 21	(13)	+−	Karp	(11)	+−
	Gilliam	(8)						
	Buie (case 9)	(8)						
	Volner	(10)						
Volner	Seerangayee	(10)						

* +− indicates a slightly positive or questionable result.
+ indicates a positive reaction and a partial or close relationship.
++ indicates a positive reaction equivalent to that obtained with the homologous material; by this method the agents are indistinguishable.
‡ Numbers indicate references cited in Bibliography.

tion of vaccine by two different methods (16, 17). Suspensions of tissue of the 27th to 41st yolk sac passages were used in the current studies.

The Kostival strain was recovered in November, 1943, from a patient in the Dobadura area of New Guinea by Blake and his coworkers (6). It also has been employed in earlier studies of scrub typhus vaccine in this laboratory (17). The 101st to 128th yolk sac passages of the Kostival strain provided material for the present work.

The mite 21 strain (also referred to as host 21) (13, 14) was isolated in mice during Novem-

ber, 1943, by Blake and his associates from a pool of mites (*T. fletcheri*) which were obtained from a bandicoot caught in the Dobadura area, New Guinea. Blood from a mouse of the sixth passage was injected into embryonated eggs (6) and yolk sac material from the 6th to 24th subcultures of this line was employed in the current work.

The wild rat 235 strain (also referred to as wild rat 2 (14)), was obtained during April, 1944, by Kohls and his coworkers (7) from the pooled brains of four wild rats (*Rattus concolor browni*) which were trapped in the Dobadura area of New Guinea. A pool of liver and spleen tissue from mice of the fourth passage served as challenge material throughout the present experiments.

The Pescadores strain was originally isolated in 1935 (3) from a native of the Pescadores Islands, and has been extensively employed in Japan (3, 20). This agent, which was temporarily lost by the Japanese, was recovered by them in December, 1945, from an axillary lymph node of a paretic who had been infected for therapeutic purposes with the strain 5 months previously (21). This reisolation was accomplished in a Mongolian hamster (*Meriones unquiculatus* M. Edward) and the agent subsequently was passed to mice wherein it showed all the usual characteristics of a strain of *R. tsutsugamushi*.[1] Portions of a suspension of pooled liver and spleen tissues from mice of the sixth passage made in this laboratory were used as challenge material throughout the present work.

The Seerangayee strain was recovered from a patient with scrub typhus in Malaya in 1934 (22). This agent was taken to Australia by Dr. R. Lewthwaite several weeks before the fall of Singapore in 1942. It had been carefully compared with other Malayan and Sumatran strains before World War II and therefore assumes considerable importance in relating pre- and postwar phases of scrub typhus investigation. The characteristics of recent passages of this strain in various hosts have been redescribed in some detail (8). The agent was brought to this country as infected yolk sac tissue in February, 1944, and was received at the Army Medical Department Research and Graduate School, from the National Institute of Health as infected guinea pig tissue. Subsequent passages were made in mice, and frozen suspensions of pooled liver and spleen tissue from mice of the second and third passages at the School provided challenge material for the present studies.

The Volner strain (also referred to as J.H.V. (23)) was recovered in 1945 in mice inoculated with blood from a patient on the island of Samar in the Philippines (23). Suspensions of pooled liver and spleen tissue from mice of the sixth and seventh passages were employed as challenge material in the current experiments.

Laboratory Animals.—Swiss mice of the Bagg Farm stock weighing 18 to 21 gm. were employed for all titrations and vaccination experiments. White rats which weighed 125 to 225 gm. were used to furnish infected tissues for the preparation of vaccines. Six or seven day old embryonated eggs were employed for the maintenance of strains and the preparation of highly infectious seed inocula for the rats. These eggs were inoculated into the yolk sac and were subsequently maintained at a temperature of 35°C. until harvested; *i.e.*, when the embryos were moribund 7 to 9 days later.

Preparation of Vaccines.—Vaccines were prepared from infected white rat tissues as previously described (16, 19); a résumé of this method follows. Freshly prepared suspensions of yolk sac tissue rich in rickettsiae were cleared of coarse particles and large fat droplets by light centrifugation; 2.0 cc. amounts were then injected intravenously into white rats. When the majority of the 20 animals inoculated with a given seed material were moribund, *i.e.* some-

[1] *R. tsutsugamushi* has been recovered from the brains of white rats from 14 to 98 days after initial infection (7) and Major B. L. Bennett, Sanitary Corps, of this laboratory has reisolated scrub typhus organisms from the spleens of mice which had been infected 10 months previously.

time during the 4th to 6th days, the lungs and spleens of recently dead and of sick rats were removed with sterile precautions, pooled, weighed, and then ground in a mortar with a small quantity of alundum. A 10 per cent suspension of the infected tissue was prepared from this triturated material using a solution consisting of 98 parts of 0.9 per cent NaCl solution and two parts of McIlvane's buffer (pH 7.4). The resultant suspension was cleared of coarse particles by sedimentation at 1,500 R.P.M. for 5 minutes in a horizontal centrifuge and the supernatant fluid was withdrawn and saved. Immediately after samples had been removed for infectivity tests, sufficient U.S.P. formaldehyde solution and merthiolate were added to the suspension to bring their final concentrations to 0.1 and 0.01 per cent, respectively. Such tissue suspensions, after storage for several weeks at 5°C. to permit inactivation of rickettsiae, constituted the vaccines; none were infectious when employed in immunity studies. Frequently several batches of vaccine, which were prepared from a single strain of scrub typhus rickettsiae, were pooled.

Vaccines from four strains of *R. tsutsugamushi* were prepared by this method. A number of these failed to meet the criteria previously established (16) for a potent antigen and were not employed in the current work.

Determination of Infective Titers.—Serial tenfold dilutions of the suspensions of infected tissues were prepared in skimmed milk medium (Difco, pH 7.2) and each mouse in a group of five to ten animals was inoculated intraperitoneally with 0.2 cc. of one of the test dilutions. The animals were observed for 21 days and deaths recorded. All mice which succumbed earlier or later than the expected time of death were autopsied; if they had no pathological evidence of experimental scrub typhus infection they were dropped from the group. In addition, the cause of death was determined at autopsy in representative animals which succumbed following typical disease in the groups which received dilutions of rickettsiae in and near the end point range. From the accumulated deaths and survivals of the test animals the 50 per cent lethal end points were calculated by the method of Reed and Muench (24).

Immunogenic Assay of Vaccines.—The capacity of a given vaccine to induce resistance to the homologous and heterologous strains of *R. tsutsugamushi* was determined in the following manner. Batches of 150 to 500 mice were segregated according to sex in groups of eight or ten per jar and each mouse was given an intraperitoneal injection of 0.5 cc. of vaccine on three occasions at intervals of 5 days. Two weeks after the final injection of vaccine, groups of eight or ten mice were challenged by the intraperitoneal injection of 0.2 cc. amounts of an appropriate dilution of a previously standardized suspension of infectious material which had been stored at −70°C. Following challenge inoculation immunized animals, as well as control mice of approximately the same weight which received similar infectious material, were observed for 3 weeks and deaths were recorded. The infectious titers of the rickettsial material in control and test mice were calculated as described above. The degree of immunity induced by a given vaccine against a strain of *R. tsutsugamushi* was expressed as an immunity index. This represented the algebraic difference between the logarithms of the infectious titers determined in control and vaccinated mice which had received comparable challenge material.

EXPERIMENTAL

Scrub typhus vaccines prepared from the four selected strains of *R. tsutsugamushi*, which appeared suitable for the purpose, were tested for their capacity to immunize mice against infections with a number of strains of the agent. The latter were chosen as representative of the species as recovered from different hosts in various geographic areas. The results of these experiments provide data which bear directly on studies dealing with the immunization of man against this disease.

Resistance of Mice Immunized with Scrub Typhus Vaccines to Infection with Eight Strains of R. tsutsugamushi.—Groups of mice were immunized with vaccine prepared from tissues of rats infected with the Imphal strain and were subsequently challenged intraperitoneally with suspensions of infectious material containing 1 to 100,000 or 1,000,000 lethal doses (LD) of *R. tsutsugamushi* of the Imphal strain or of one of seven other strains of scrub typhus. At this time normal mice of the same weight as the vaccinated animals were injected with portions of each challenge suspension. Results illustrative of those obtained in a typical experiment are summarized in Table II. The data are presented in detail in order to indicate the type of mortality distribution usually found in mice which possess different degrees of resistance to infection with scrub typhus. Elsewhere in this report tabular data are given in a more condensed form and include only values for the 50 per cent lethal titers in normal and vaccinated mice and the immunity index; however, each index is based on titrations similar to those illustrated in Table II.

TABLE II

Resistance of Mice Immunized with Imphal Vaccine to Challenge Infection with Strains of R. tsutsugamushi

Rickettsial strain	Vaccinated mice*	Challenge inoculation: Dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Infective titer 50 per cent (lethal)	Immunity index‡
Imphal 8	Controls		10/10	10/10	9/10	10/10	9/10	4/10	1/10	0/10§		$10^{-6.8}$	
	Vaccinated	7/8	8/10	8/10	4/10	1/10	0/10	0/10	0/9			$10^{-3.7}$	3.1
Imphal 8	Controls		10/10	10/10	10/10	9/10	3/9	7/9	2/10	0/10		$10^{-7.0}$	
	Vaccinated		7/8	5/10	5/10	1/9	2/8	1/10	0/8			$10^{-3.8}$	3.2
Karp	Controls				10/10	10/10	10/10	10/10	4/10	3/10	0/10	$10^{-8.1}$	
	Vaccinated			10/10	10/10	8/10	8/10	4/10	5/10	0/9		$10^{-6.9}$	1.2
Seerangayee	Controls				10/10	9/9	9/10	4/10	1/9	0/10		$10^{-6.8}$	
	Vaccinated				10/10	8/10	9/10	3/10	1/10			$10^{-6.6}$	0.2

* Mice were immunized with vaccine 1-2-5 prepared from white rats infected with Imphal 8 strain; a summary of this data is included in Table III.

‡ Immunity index represents the difference between exponential values of the infective titers in the contro and test groups.

§ Numerator indicates the number of mice in each group that died of the challenge inoculum and the denominator indicates the number of mice in the group.

A summary of experiments done with Imphal vaccines is found in Table III. It is evident that the present Imphal vaccines were comparable in potency to those prepared previously (16); they protected mice against 1,000 LD of the homologous strain. Such vaccinated mice displayed a variable degree of resistance when challenged with heterologous strains of *R. tsutsugamushi;* how-

ever, in no instance was the immunity index as great as when the Imphal strain was used. It does not appear feasible with the data available to attempt to arrange sharp levels of immunity when considering resistance to heterologous strains. Instead it seems preferable to consider the results as indicating a broad spectrum with various levels of cross-protection, each of which shades into the other. Thus, Imphal vaccine induced appreciable resistance to the Karp and Kostival strains, slight to wild rat 235, but none to Seerangayee, Pescadores, or mite 21. While the results in general were consistent, divergent data were obtained in the two experiments in which the Volner strain was used

TABLE III

Resistance of Mice Immunized with Imphal Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1–2			Vacc. 3–4			Vacc. 1–2–5		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Imphal 8	6.1‡	2.5	3.6	6.9	3.4	3.5	6.8	3.7	3.1
Imphal 8				5.8	2.7	3.1	7.0	3.8	3.2
Karp				8.2	6.0	2.2	8.1	6.9	1.2
Kostival	6.7	4.4	2.3	7.1	5.4	1.7			
Wild rat 235							4.3	3.2	1.1
Volner				6.0	3.9	2.1	5.9	5.4	0.5
Seerangayee				6.8	6.5	0.3	6.8	6.6	0.2
Pescadores							7.2	7.2	0.0
Mite 21				6.6	6.7	−0.1			

* See footnote to Table II.

‡ The exponential values of the 50 per cent lethal end point titers are expressed as positive numbers.

for challenge (Table III). It is not unlikely that in the zone of partial immunity secondary factors such as the general health of the test mice prior to challenge have considerable effect on the final result and may account for such discrepancies.[2]

Mice immunized with vaccines prepared against the Karp strain were more resistant to infection with the homologous organism than to infection with the heterologous strains (Table IV). It is evident from the tabular data that the

[2] Vaccinated animals are not solidly immune to challenge even with the homologous agent. They frequently show signs of active infection which persist for a number of days, and during periods of illness the margin of vitality between death and recovery is narrow. The use of occasional groups of mice with inapparent enzootic infections in certain of our experiments may have weighted the balance toward death of the mice and this resulted in lower indices for the test assay.

two vaccines which had indices above 3.0 with the homologous strain afforded some protection against infection with Volner, Kostival, and Imphal strains, little if any against the Seerangayee, and none against mite 21 strain. It is of interest to compare these results with those obtained with vaccine lot 8-9-10-11, which had indices of 2.0 and 0.9 in duplicate tests with the homologous strain (Table IV). This relatively poor vaccine[3] induced no resistance to any of the heterologous strains.

The results of experiments with Kostival vaccines are summarized in Table V. It is evident that the vaccine protected as well against wild rat 235 as against the homologous strain. Furthermore, it induced considerable resist-

TABLE IV

Resistance of Mice Immunized with Karp Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1			Vacc. 6-7			Vacc. 8-9-10-11		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Karp	8.6*	4.6	4.0	7.9	4.7	3.2	7.9	5.9	2.0
Karp				8.0	4.6	3.4	7.9	7.0	0.9
Volner				6.6	4.2	2.4	6.7	6.6	0.1
Kostival	7.9	6.2	1.7	7.2	5.2	2.0	7.3	7.1	0.2
Imphal 8				5.8	4.1	1.7			
Seerangayee				7.4	6.4	1.0	6.9	6.6	0.3
Mite 21				6.7	6.5	0.2			
Pescadores							7.0	7.1	−0.1
Wild rat 235							3.2	3.7	−0.5

* See footnotes to Tables II and III.

ance to infection with Imphal, Karp, and Volner organisms but none against Seerangayee, mite 21, or Pescadores. Here again some variation occurred in the indices obtained with given heterologous strains in experiments with different batches of vaccine, nevertheless, the trends of the results were consistent.

Pertinent data on the experiments in which mite 21 vaccines were employed are summarized in Table VI. It is immediately obvious that none of the mite 21 preparations was able to induce appreciable resistance to infection with

[3] This pooled material had been stored in the fluid state at 5°C. for about 6 months when the assay was begun; each of its constituent vaccines had been prepared from tissues having a sufficiently high infective titer to suggest that the immunogenic potency would be satisfactory (16). Because of the low potency of the pool we assumed that deterioration had occurred during storage. The results of controlled tests (18) indicated the validity of this assumption and showed that stability on storage could be attained with greater regularity when scrub typhus vaccines were lyophilized.

the mite 21 strain. Nevertheless, these materials were tested with heterologous strains for two reasons: (1) each lot had been prepared from highly infectious material, titer $10^{-7.5}$ to $10^{-8.2}$, and hence contained amounts of rickettsial substances which we thought should have been sufficient to immunize, and, (2) none of the vaccines prepared from Imphal-, Kostival-, or Karp-infected tissues induced even slight resistance to infection with the mite 21 strain. The results were nonetheless unexpected in that the mite 21 vaccines induced as great or greater resistance to infection with wild rat 235, Kostival, Imphal, and Karp strains than to the homologous organism. Indeed, respective indices of 3.5

TABLE V

Resistance of Mice Immunized with Kostival Vaccines

Challenge rickettsial strain	Mice injected with											
	Vacc. 2			Vacc. 5-6			Vacc. 7-8			Vacc. 9-11		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.		Control	Vacc.	
Kostival.....	7.2*	3.9	3.3	6.7	3.0	3.7	7.2	3.4	3.8	7.7	5.6	2.1
Kostival.....				6.5	4.0	2.5						
Wild rat 235...							5.2	1.6	3.6			
Imphal.....				6.1	3.3	2.8				6.9	5.2	1.7
Karp.....	8.4	5.8	2.6	7.7	6.7	1.0						
Volner.....				6.0	4.1	1.9	6.4	5.7	0.7			
Seerangayee...				6.7	6.3	0.4						
Mite 21.....				6.7	7.0	−0.3	6.9	6.9	0.0			
Pescadores....							7.5	7.7	−0.2			

* See footnotes to Tables II and III.

and 3.2 were obtained with the wild rat 235 and Kostival strains in mice immunized with vaccine 4-5 while values of 0.9 and 1.1 resulted in the duplicate test in which the mite 21 strain was used for challenge. In the single experiments in which the Pescadores, Volner, and Seerangayee strains were used for challenge no resistance was noted.

Summary of Results of Cross-Vaccination Experiments.—In order to facilitate a comparison of the antigenic relationships established by the present cross-vaccination experiments, an interpretative summary is given in Table VII. Only those Imphal, Karp, and Kostival vaccines mentioned in Tables III to V which had immunity indices of 3.0 or greater with the homologous organism were considered in compiling the summary. Since none of the mite 21 vaccines gave a high index value with the homologous strain, no selection was applied to the data in Table VI. Indices obtained in several different tests with comparable materials were averaged and when such values were 2.5 or greater the

degree of cross-reaction was arbitrarily designated as ++, similarly, average values from 1.5 to 2.4 were designated as +, values of 0.5 to 1.4 as + −, and those of less than 0.5 as zero for Table VII.

TABLE VI

Resistance of Mice Immunized with Mite 21 Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1			Vacc. 2-3			Vacc. 4-5		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Mite 21	7.0*	6.3	0.7	6.7	6.2	0.5	7.2	6.3	0.9
Mite 21				7.8	6.4	1.4	6.8	5.7	1.1
Wild rat 235							5.0	1.5	3.5
Imphal							6.5	3.7	2.8
Kostival				7.0	5.5	1.5	7.9	4.7	3.2
Karp	8.2	7.3	0.9	8.2	6.5	1.7	8.1	6.4	1.7
Pescadores							7.1	6.5	0.6
Volner				6.4	6.3	0.1			
Seerangayee				6.8	6.9	−0.1			

* See footnotes to Tables II and III.

TABLE VII

Interpretative Summary of Results of Cross-Vaccination Tests with Strains of R. Tsutsugamushi

Challenge rickettsial strain	Vaccine			
	Imphal	Karp	Kostival	Mite 21
Imphal	++	+	++	++
Karp	+	++	+	+−
Kostival	+	+	++	+
Wild rat 235	+−		++	++
Volner	+−	+	+−	0
Mite 21	0	0	0	+−
Pescadores	0		0	+−
Seerangayee	0	0	0	0

See text for explanation of table.

The data indicate that the Imphal, Kostival, and Karp strains are rather closely related to one another. Furthermore, each of these has one or more immunogenic factors in common with the Volner strain and the first two, at least, also possess components similar to those occurring in wild rat 235. The mite 21 strain contains antigenic structures found in the Imphal, Kostival, Karp, and wild rat 235 organisms, and, in addition, a factor common to itself

and the Pescadores agent. However, the mite 21 organism is peculiar in that vaccines prepared from it appear deficient in substances necessary for inducing good protection against itself. It is particularly striking that vaccines against none of the four agents induced resistance to the Seerangayee organism, hence on the basis of this method of testing, these strains appear entirely unrelated. Furthermore, it is apparent that there is no correlation between the geographical origins of the agents employed (see Materials and methods) and the relationships established by these studies.

Efficacy of a Bivalent Vaccine.—Since the monovalent vaccines prepared from four strains of *R. tsutsugamushi* each failed to induce protection against certain strains of this agent, it appeared desirable to test the immunizing capacity of a

TABLE VIII

Resistance of Mice Immunized with a Bivalent (Karp-Mite 21) Vaccine

Challenge rickettsial strain	Mice injected with								
	Monovalent Karp (diluted 1:2)			Monovalent mite 21 (diluted 1:2)			Bivalent Karp and mite 21		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Karp	8.2*	5.5	2.7				8.2	5,7	2.5
Mite 21				7.4	6.6	0.8	7.4	6.5	0.9
Seerangayee							6.5	6.3	0.2

Karp vaccine 12 and mite 21 vaccine pool 4-5 were mixed in equal proportions for the bivalent vaccine. Each monovalent vaccine diluted with an equal volume of saline was used for a comparative test.

* See footnotes to Tables II and III.

bivalent vaccine. Accordingly, a mixture of equal volumes of a mite 21 and a Karp vaccine was used to immunize a group of mice. In addition, other groups of mice received the mite 21 or Karp vaccine diluted with equal volumes of physiological saline solution. Subsequently the animals which had been injected with the diluted monovalent vaccines were challenged with the homologous organism while those immunized with the bivalent vaccine were divided into three groups and infected with either mite 21, Karp, or Seerangayee strains of rickettsiae. The results of this experiment are presented in Table VIII.

It is evident that resistance induced by the bivalent vaccine was essentially the same as that induced individually by each of its component parts. Since neither component alone elicited immunity to the Seerangayee strain, it was to be expected that the mixture would also fail to immunize against this heterologous strain. The same reasoning might explain the lack of augmentation of resistance to the mite 21 agent in the mice receiving the mixture. However, it had been anticipated that the bivalent material would, at least, provide

increased protection against Karp since the results of earlier experiments had indicated that mite 21 vaccine induced some immunity to the Karp strain.

Resistance of Convalescent Mice to Inoculation of Homologous and Heterologous Strains of R. tsutsugamushi.—It has been demonstrated repeatedly that animals infected with one strain of *R. tsutsugamushi* are resistant to infection with heterologous strains of this agent. Each of the strains used in the present work had been previously tested in this respect and found to respond in the usual manner (see Table I, under Materials and methods). Nevertheless, in view of the differences shown to exist among the eight strains by means of the cross-vaccination tests discussed above, it seemed desirable to recheck the identity of the strains at the termination of the present study. Accordingly, mice convalescent from infection induced by the subcutaneous injection of small amounts of Kostival rickettsiae were segregated in groups of five and injected intraperitoneally with 10, 100, or 1,000 minimal lethal doses of organisms of the Kostival, Imphal, Karp, mite 21, Volner, Pescadores, or Seerangayee strains. Convalescent animals survived inoculation with the various challenge materials, hence, all of these strains appeared characteristic of *R. tsutsugamushi* when reexamined in a cross-immunity test.

DISCUSSION

Formalin-inactivated vaccines prepared from tissues of rats infected with the Imphal, Karp, Kostival, or mite 21 strains of *R. tsutsugamushi* induced resistance in mice against infection with the homologous as well as certain heterologous strains of the organism. However, none of the vaccines was capable of eliciting protection against all eight strains of *R. tsutsugamushi* employed in the present work. These results, which add evidence to that already obtained by other methods (11–14), warrant the conclusion that significant differences in antigenic structure exist among the organisms now classified as *R. tsutsugamushi* even though infection with one strain renders animals immune to reinfection with other strains of the agent. Despite these differences, at the present time it does not appear desirable to indulge in taxonomic speculation regarding division of the subgenus *R. tsutsugamushi.* However, it may be noted that while cross-immunity is exhibited by animals which recover from infection with *R. prowazeki* and *R. typhi*, or from infections with *Dermacentroxenus rickettsi* and *D. coroni*, nevertheless, vaccines prepared against one member of the pairs of agents afford comparatively little protection against the other (25–27).

The present observations suggest that certain strains of *R. tsutsugamushi* may be more broadly antigenic than others. If this is true, then a search is warranted for an organism possessing the entire antigenic mosaic of the subgenus since it might yield a vaccine capable of protecting man against naturally occurring infection with scrub typhus. It should be borne in mind, however, that those strains which appear to possess more antigenic constituents than

others may consist actually of a mixture of several strains. Such mixtures might be obtained unwittingly in the laboratory by combining infectious material from several sources. Thus, the Imphal 8 strain was derived from the pooled bloods of five patients and the mite 21 agent from a pool of over 250 mites. Furthermore, the possibility exists that the rickettsiae recovered from a single naturally infected rodent may represent a biological mixture of strains. This idea receives some support from the observations of Audy (28) on one of the mite vectors in the Imphal area; he states, "... in many populous colonies of wild rats the turnover of *Trombicula diliensis* may well amount to some 5,000 larvae per rat per annum." The possibility of a dual infection in such an infested rat is not unlikely.

CONCLUSION

Antigenic differences among strains of *R. tsutsugamushi* are sufficiently great that vaccines prepared from certain strains fail to induce resistance in mice to infection with other strains. Although the results of cross-vaccination tests indicate varying degrees of relationship between a number of the strains, there is no correlation between source of the rickettsia and antigenic pattern of the agent.

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FOOD PROTEIN EFFECT ON PLASMA SPECIFIC GRAVITY, PLASMA PROTEIN, AND HEMATOCRIT VALUE*

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We shall show that the amount of protein eaten by normal individuals alters the specific gravity of their plasma and the hematocrit value of their blood. A point of particular interest is that these two changes move in opposite directions. The specific gravity rises but the hematocrit value falls as the protein intake increases from low but adequate, through moderate, to high but not excessive levels.

For clinical purposes a rise in specific gravity is taken as an index of a rise in the protein concentration of the plasma. It might be supposed, therefore, that in these experiments, an increase in protein consumption had been followed by a rise in plasma protein concentration. In that case a plausible explanation for the opposite movement of the hematocrit value is available. Since a rise in plasma protein concentration is frequently followed by an increase in blood volume, the fall in the hematocrit value may be regarded as a secondary phenomenon consequent to a dilution of the blood in respect of its red cell concentration.

The analysis of variance was applied to the data. The factors postulated in these experiments were thus defined in regard to their relative importance and in regard to some of their interrelations. Finally it was shown that the rise in specific gravity and the fall in hematocrit value, as protein consumption increased, could not reasonably be ascribed to chance.

The hypothesis that the changes in specific gravity and hematocrit values are causally related takes it for granted that the rise in specific gravity was due to an increase in protein concentration. This is, perhaps, a doubtful assumption, particularly when protein consumption is increased, for the observed degree of change in specific gravity might conceivably arise from changes in other constituents of the plasma than protein. Direct plasma protein measurements during a reiteration of the experiment would have answered the question. But since, for various reasons, this repetition was not feasible, we had resource to a similar experiment on rats. It was found that the protein concentration of the rats' serum rose, much as the plasma specific gravity rose in the human experiments. However in the rats there was no corresponding fall in the hematocrit readings. Thus the suggested explanation of the

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inverse relation between the specific gravity and hematocrit measurements in man is left as an, at best, unconfirmed hypothesis. This explanation became still less plausible when it was found that both serum protein concentration and packed cell volume may rise when the food contains no protein. The facts, however, remain and have, in themselves their own limited and empirical value[1].

Conditions and Methods

The first observations were made in 1940 when 10 residents and internes collaborated with us in trying to answer a question relating to renal function. During 3 successive weeks they took 0.5, 1.5, and 2.5 gm. of protein per day per kilo of body weight. The food in which these quantities of protein were given was calorically adequate. There was no limitation of water or salt consumption. During each week the diets were taken from Monday to Friday inclusive, while on Saturdays and Sundays no restrictions of any sort were imposed.

In 1940 we were beginning, in our out-patient clinical work, to use an approximate and rapid hematocrit method based on centrifuging blood at a constant speed for a constant time. We needed, from normal individuals, a measure of the variability of the measurements obtained with this method, and we used the 60 samples of blood obtained from our subjects for this purpose. The collections were made at 11:45 a.m. on the 4th and 5th days of each of the three diets. The blood was drawn into a vaselined syringe. Six ml. of well mixed blood was transferred to a cylindrical graduated tube containing 12 mg. of dry potassium oxalate. After inversion to ensure solution of the oxalate, the tube was centrifuged for exactly 5 minutes in a centrifuge driven by an induction motor that ran at 3500 R.P.M. The percentage of the total volume occupied by red cells was taken as the hematocrit value. For the specific gravity measurements we used Kagan's method (1) which is an adaptation of the falling drop technique introduced by Barbour and Hamilton (2).

The second series of observations was made in 1945, on a group of 10 medical students (9 men and 1 girl) under the same dietary conditions. After an interval of 2 months, the results were checked by repeating the observations on the 0.5 and the 2.5 gm. protein per kilo body weight diets. The uniformity of the increase in urea excretion as the protein consumption rose indicated that there was no significant difference between the dietary conditions to which the 1940 and the 1945 groups were subjected. The observations were made at different times than in 1940, namely on the 5th day of each diet, and 4 collections of blood were taken at 7:15 a.m., 11:45 a.m., and 4:45 p.m., just before breakfast, lunch, and dinner; and at 9:45 p.m., just before going to bed.

The standard Wintrobe method (3) was used in 1945 with the hematocrit, and for the specific gravity the copper sulfate method introduced by Phillips and his associates (4). In the subsequent experiments on the rats, the hematocrit determinations were made on the first blood obtained by severing the abdominal aorta. Potassium oxalate, 2 mg. per 1 ml. of blood, was used as an anticoagulant, but, thereafter, Wintrobe's technique was followed. The protein concentration of the serum was measured by a rather tedious and time-consuming gravimetric method, the details of which have been summarized by Barrett in a paper on another procedure (5). This is the method we used in determining the significance of the colors developed in the biuret measurements employed in the experiments we report on the effect of very low protein diets in man. In a considerable experience over many years with

[1] The work was divided so that one author (T. A.) measured the specific gravity and hematocrit values, another (H. G.) was responsible for the statistical analysis, while the third (E. B.) made the gravimetric and biuret determinations of the protein content of the serum or plasma.

many devices for the determination of protein, we have been forced to the conclusion that the ancient and laborious gravimetric method is still the most reliable and the only one we can depend on as a standard.

RESULTS

The 59 specific gravity and hematocrit measurements made in 1940 are too small in number for an adequate analysis of the various factors involved, and this precludes a complete comparison with the 1945 data, wherein we have 189 specific gravity and 198 hematocrit determinations. In any case, however, the existence of a pronounced diurnal variation restricts us to a comparison of observations made at the same time of day. Thus only the data obtained at 11:45 a.m. can be compared, and of these only those obtained on the 5th day of the diet are strictly comparable.

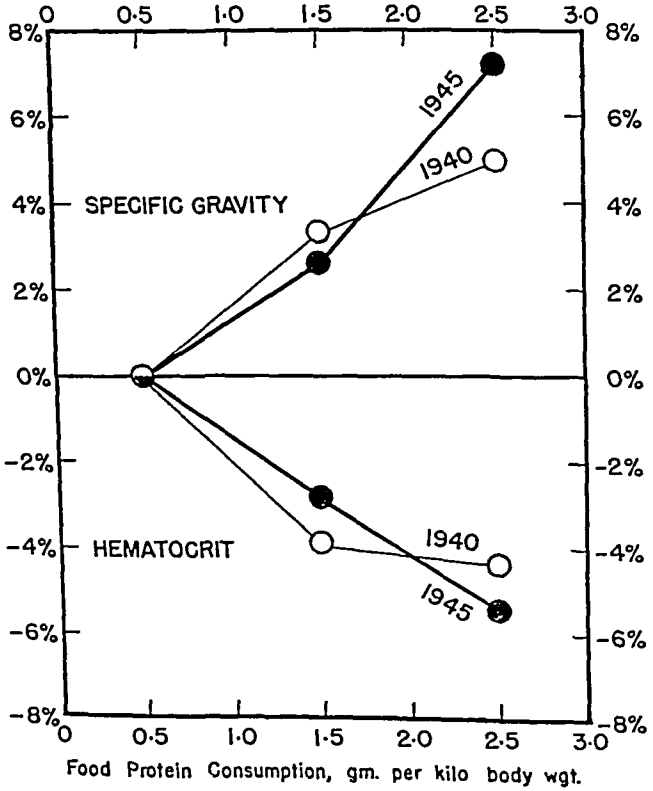

FIG. 1. Comparison of the relative changes in specific gravity observed at 11:45 a.m. on the 5th day of each diet in 1940 and in 1945. Expressed as percentage changes from the means on the 0.5 gm. protein per kilo body weight diets.

Fig. 1 compares these few data from 1940 and 1945 expressed in percentage changes from their mean values on the 0.5 gm. diet. In both experiments,

though the methods were different and the subjects were not the same, the specific gravity rises and the hematocrit falls as the protein intake is increased.

TABLE I

Mean Specific Gravity and Hematocrit Values

1910 experiment		
Protein intake	Specific gravity (Kagan) as protein	Hematocrit value 5 min. at 3500 R.P.M.
gm. per kilo body weight	*gm. per 100 cc.*	*per cent*
0.5	6.8	50.0
1.5	6.9	47.9
2.5	7.2	47.1
1945 experiment		
Protein intake	Specific gravity ($CuSO_4$) as protein	Hematocrit value 35 min. at over 3000 R.P.M.
gm. per kilo body weight	*gm. per 100 cc.*	*per cent*
0.5	7.4	46.6
1.5	7.6	45.5
2.5	8.0	43.6

In Table I, we give the means of all the observations made in 1940 and 1945. The absolute values in the two experiments are not comparable because of time relation differences and because the hematocrit specimens in 1940 were not centrifuged to complete packing. Relatively however, the change in both cases is similar, for as the protein intake is increased the specific gravity rises and the hematocrit value falls.

Interpretation of the Results According to the Analysis of Variance

The data given in the first part of this paper have been tested by the method known as the analysis of variance. Apart from the intrinsic value of the conclusions thus validated, it may be hoped that other clinical investigators, whose results are often obtained under conditions similar to those observed in the present experiments, may find a use for this method in the treatment of their own data. The analysis is presented as an illustration of the fact that more information as to the meaning of a series of measurements can be obtained from the analysis of variance than is possible by the use of the older methods that are still almost exclusively used in the treatment of the results obtained in clinical investigation.

The idea underlying the continued use of the older methods may be described as the "crucial experiment" doctrine. For long this has been insisted on as the invariable scientific approach. It purports to test putative causes

one at a time, on the assumption that laboratory workers can control all other factors than the hypothetical one with assurance, even with certainty. Historically, this notion derives from the 19th century, when it was thought proper to extend the assumptions underlying work in Newtonian physics to cover the treatment of biological measurements.

This doctrine, so long held an indisputable though difficult ideal, has in time been undermined by two forces. The first is the frequency of controversy when a reported crucial experiment is repeated under supposedly identical conditions but a different result is obtained. Each expert is then inclined to believe that he controlled the essential factors and even to assert that the other failed to do so. This attitude involves the assumption of a single factor law of cause and effect, although this visionary simplicity is non-existent. On the contrary, biological problems even more than physical, involve multiple factors which are much less easy to control or even exclude than is supposed by crucial experimenters. For many years it has been evident that the crucial experiment is an ideal trouble-maker, though no other principle of experimental approach has received general acceptance in clinical experiments. The crux still exerts its dead hand.

The second undermining force is the variability of nearly all human factors, even under standardized conditions. Of recent years, this difficulty has been solved for many situations by the work of mathematicians, for they have increasingly simplified and extended the rules of probability to small samples, say 30 repetitions of an experiment, or even less. So in biological and medical papers the age-old average and range of variation are now supplemented by some measure of variability. This has permitted precise statements as to whether the difference between observed means was attributable to chance or was significant. All this has led to a weakening of the doctrine of the crucial experiment.

The further advance with which we want to deal here, permits a working hypothesis that a postulated factor is primary, but it provides evidence which will confirm that postulate or alternatively deny it in favor of some other factor in the original hypothesis, visualized as secondary or possibly one hardly considered. Furthermore the method affords evidence as to the degree to which the factor which proves primary is not only accompanied but itself influenced by other factors.

Fisher's analysis of variance, designed originally for the simultaneous testing of multiple factors during experiments in agriculture, is sometimes appropriate to experiments in man. This method involves the splitting of the total variability and the assignment of the several fractions to the factors responsible for their production.

The classical exposition is found in Fisher (6) and useful practical guides to the computations and interpretations in Snedecor (7). Here it is impractical

to do more than give a concrete illustration of the use of the method in the analysis of the data given in this paper.

The working hypothesis in our experiment was the supposition that in normal individuals a change in protein consumption might be followed by a change in specific gravity and hematocrit measurements. As far as averages can go, this hypothesis was supported (Table I). In 1940, as in 1945, an increase in protein consumption was associated with an increase in specific gravity and a decrease in hematocrit readings, in different subjects and in measurements made by different methods. Furthermore, in both experiments the use of the conventional methods indicates that the changes are "significant;" that is, that there is only a remote possibility that they were a consequence of chance variation.

It would be an error, however, to assume that the variation in protein intake was the only factor involved. The results are influenced significantly in both 1940 and 1945 by variation amongst the subjects of the experiments, and in the 1945 experiment by the time of day at which the measurements were made. In addition it is shown that in both experiments the measurements were altered by a synergistic combination of the effect of the protein intake and the effect of the individual differences amongst the subjects. This is known technically as an "interaction." It is indicated by the fact that the protein variation was followed by a greater effect in some subjects than in others. These interactions are multiple. Thus for the 1945 experiment we have estimated the effect of the interactions between the protein and the times at which the observations were made, and between the protein and the subject, and have measured the effect of a triple interaction between the protein, the subject, and the time. That, in addition to these reactions, other factors than those we have examined were operative is certain. These, however, will suffice to indicate the scope of the method.

Tabulations of the condensed analysis of variance are put on record for those interested in such documentation, in Tables II and III. With reference to Table II it may be noted that discrepance = subclass discrepance, or between days within subjects, or, in less technical language, between replications on each patient on different days. This discrepance is the residual error which we have been unable to analyze into smaller parts; it is therefore to be taken as the basic unavoidable variability or error. With it we compare the variability due to each factor stated; for example, protein intake. The variability is examined in terms of the mean square, and most conveniently by the F ratio; *i.e.*, the MS to be tested, divided by the error-MS. In this example, we have $46.5/2.3 = 20$, and entering Snedecor's table with $F = 20$, and with the proper degrees of freedom here 2 and 30, we read the probability; it turns out less than 0.01; *i.e.*, the 1 per cent level: this means that the chance of finding a variability (mean square) as large as here, would be less than one in a hundred repetitions of this experiment; a chance so small that it is customary to ignore it and say the result is significant.

With reference to Table III, the computations revealed the operation of several 2-factor interactions. This indicated the necessity of tabulating the complete analysis, instead of

reporting only the simple form which covered the fractions of variance found significant in the 1940 experiment. Then the non-significance of the interaction Times × Subjects and of Proteins × Subjects × Times, warranted the combination of these (lines 6 and 7) into a pooled error variance (line 8). Finally this last item was used as the basis of reference to demonstrate the significance of items 1 to 5, as already stated in general terms. The computations to demonstrate significance, by the *F* test, are not reproduced; the method is the same as illustrated for Table IV; here the protein intake variability is tested; 28.3/0.63 = 45; and entering Snedecor's table, we again find this value statistically significant.

What proportion of the total variability is attributable to each of these three main factors? This simplification is not customary, but as a rough estimate the following items in the tables may be pointed to. In the 1945 experiment, for the hematocrit readings, the subjects contributed by far the largest proportion of the total sum of squares: 1581/1924 = 82 per cent, the time factor contributed 181/1924 = 9 per cent, but the protein level contributed only 57/ 1924 = 3 per cent; with minor contributions by the various interactions.

Similarly in 1945, for specific gravity, the subjects contributed 10.25/21.03 = 49 per cent of the total sums of squares, the protein level contributed 2.70/21.03 = 13 per cent, and the time element contributed 1.21/21.03 = 6 per cent; with minor contributions by the interactions.

The subjects made much the most notable contributions; but for the other two factors the order was not the same. Hematocrit readings seemed influenced by time factor more than by protein intake, whereas for specific gravity the protein was the more important factor.

The essence of the analysis distils out as follows:—

Subject Factor.—This was markedly significant by the statistical test, and in general was the most potent factor.

Protein Factor.—This was also markedly significant.

Time Factor.—This in the 1940 experiment was days, *i.e.* blood samples on 2 successive days; it caused no variability, either in hematocrit readings or

TABLE II

1940 Experiment

Source of variation	Degrees of freedom, *df*	Hematocrit value		Specific gravity	
		Sum of squares, *SS*	Mean square, *MS*	Sum of squares, *SS*	Mean square, *MS*
Protein, *P*	2	93.1	46.5	1.289	0.645
Subjects, *S*	9	549.8	61.1	1.140	0.127
Interaction *P* × *S*	18	65.7	3.6	0.524	0.029
Discrepance, for error	30	69.3	2.3	1.240	0.041
Total	59	777.9		4.193	

specific gravity; and therefore the calculations are not reproduced in Table II. On the other hand, time in the 1945 experiment was hours, *i.e.* samples at 4

different hours on the same day; this factor was a real source of variability and therefore the analysis is shown in the appropriate detail in Table III.

TABLE III

1945 Experiment

Analysis of Variance

Line	Source of variation	Degrees of freedom, *df*	Hematocrit value		Specific gravity	
			Sum of squares, *SS*	Mean square, *MS*	Sum of squares, *SS*	Mean square, *MS*
	Main effects:					
1	Protein, *P*	2	56.6	28.3	2.70	1.349
2	Subjects, *S*	9	1581.2	175.7	10.25	1.139
3	Time, *T*	3	181.0	60.3	1.21	0.403
	2-Factor interactions:					
4	$P \times T$	6	32.8	5.46	0.93	0.155
5	$P \times S$	18	21.8	1.21	2.23	0.124
6	$T \times S$	27	22.1	0.82	1.36	0.050
	Triple interaction:					
7	$P S T$	54	29.0	0.54	2.36	0.044
8	Pool 6–7, for error	81	51.0	0.63	3.71	0.046
9	Total	119	1924.5		21.03	

Does the Protein Concentration as Well as the Specific Gravity of the Plasma Increase When Protein Consumption Increases?

By virtue of the analysis of variance it is possible to derive from the data many well founded conclusions, of which the most important for our immediate purpose are the rise in specific gravity and the fall in hematocrit value. Why may we not suppose than an increase in plasma protein concentration is followed by an increase in plasma water, not enough to prevent some rise in protein concentration but sufficient to account for the fall in the hematocrit value? The advantage would be that we could then view both of these changes as a consequence of a single underlying metabolic alteration such as might well follow an increase in protein consumption.

The difficulty is that the statistical analysis supports only one of the two facts that the hypothesis purports to explain. We now know that an increase in protein intake is associated with a decrease in the hematocrit value, but it is not proved that there is any increase in plasma protein concentration. What we measured was specific gravity. Unquestionably that rises as the hematocrit value falls. But when, following clinical usage, we expressed these specific gravity changes in terms of protein, we were making an assumption

which may not be valid under the conditions of our experiments. It is this assumption that warrants a brief discussion.

We are not concerned here with the general question as to whether, for most clinical purposes, it may or may not be justifiable to predict plasma protein concentration from specific gravity measurements. We think that question has already been answered in the affirmative by the careful work of many investigators between 1930 (8) and the present day.

What we should most of all like to know is whether the increase in the specific gravity in our subjects can be accounted for by a parallel increase in protein. That knowledge we cannot get because we did not measure the protein while the experiments were under way, and various circumstances prevent us from reduplicating the conditions we once had. All that can be said now is that there are two general considerations which make us hesitate to assume a parallelism between specific gravity and protein concentration under the particular circumstances of our experiments. First, the rise of specific gravity acquires most of its statistical significance from the number of observations from which the averages were derived. The absolute change in specific gravity as we pass from 0.5 to 2.5 gm. of protein is represented in terms of protein by a rise from 6.8 to 7.2 gm. protein per 100 cc. in 1940, and from 7.4 to 8.0 gm. protein per 100 cc. in 1945. Now the general relation between specific gravity and protein amount that we find so useful in clinical work, where we rarely attempt to draw inferences unless the differences from our normal standards are larger than those we are here considering, depends mainly on the fact that the electrolyte concentration in the plasma remains relatively constant under a wide variety of conditions. Major changes in specific gravity can thus be ascribed and have, in fact, been shown to be due, in the main, to shifts in protein concentration. However in this particular experiment, we have no assurance that the non-protein organic constituents remain as constant as the inorganic. Thus, both in 1940 and in 1945, we found that the serum urea concentration more than doubled as we went from 0.5 to 2.5 gm. of protein per kilo (9) and though, in itself, this could have had no appreciable effect on our measurements, it is possible that the summation of parallel increases in other organic constituents may have been a factor in the rise of specific gravity as the protein intake was increased. Second, plasma is a mixture of substances, and we cannot, as yet, assign to each substance its part in the production of the specific gravity we measure. For that we should need a knowledge of the concentrations of all of the substances in the plasma. Each has its own specific effect and while, for most of them, an increase in their concentration will increase the specific gravity, there are others, fats for instance, that decrease specific gravity as they accumulate in the plasma. Thus a decrease in fat concentration as the protein intake rose is at least a possible reason for the observed rise in specific gravity.

Since we could not reiterate our experiments on human subjects, we had resource to a parallel experiment on rats.

We have compared the protein concentration predictions from specific gravities as determined with the copper sulfate method with actual protein measurements by the gravimetric method. The variation in protein consumption was obtained by replacing corn starch with casein so that we got three diets with the same caloric, vitamin, and mineral content, in the first of which there was no casein, in the second 17 per cent, and in the third 74 per cent of the total food weight.

There were 10 rats on each of these three diets, 5 male and 5 female, chosen so that they were of similar weight (200 gm. for the male and 180 gm. for the female), all selected from a colony living on a stock diet that contained 17 per cent of protein. The blood was obtained by serving the abdominal aorta under ether anesthesia. The observations were made 48 hours after the diets were given because we have found that at this time on these diets the major changes had occurred in the total and organ protein content of the animals (10). The results are given in Table IV.

TABLE IV

Protein consumption	Protein concentration in serum		
	Gravimetric	Biuret	Specific gravity ($CuSO_4$)
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*
Low (1 per cent protein in diet)	5.72	5.91	5.58
Medium (16 per cent protein in diet)	6.23	6.07	5.76
High (74 per cent protein in diet)	6.57	6.25	5.94

The results given in Table IV give us some reason to suppose that the specific gravity changes we observe in man when the consumption of protein is increased may be a consequence of changes in protein concentration and that they were probably not due to alterations in other constituents of the plasma. Certainly, in the rat, increase in protein consumption is followed by an increase in serum protein concentration and the direction of the change is given by specific gravity measurements.

The Mechanism of the Relation between Protein Consumption and Serum Concentration

In the experiment whose results are given in Table IV we have an example of the fact that an increase in protein consumption is associated with an increase in serum protein concentration. But this same experiment denies our original supposition that there should be a concomitant fall in the packed cell volume. The averages of closely agreeing values were 41.7 for the low, 41.5 for the moderate, and 42.5 per cent for the high protein intake. An explanation that was plausible for man is found to be inapplicable to the rat and is thus shown to be devoid of any general physiological significance. There

remains the question as to why the serum protein concentration rises and falls with change in protein consumption.

The question as to why the serum protein concentration falls when the protein consumption is sharply reduced to levels that are inadequate for nutrition has been investigated by Bloomfield (11). Using the gravimetric method, and working with large numbers of rats, he showed that all of the decrease in serum protein concentration occurred within 48 hours of the change in diet. Thereafter, over very long periods of time, there was no further significant decrease in concentration, in spite of a pronounced and continuing loss of body weight. After 147 days on the low protein diet, the serum protein concentration promptly returned to its original level when an adequate amount of protein was given. It is reasonable to assume that continued observation would have shown that it stayed thereafter at about that level. Certainly it is a fact we could support by many observations, that, after the initial increase, there is no further change in concentration, no matter how long we go on feeding adequate or even excessive amounts of protein.

Bloomfield noticed that the rapid shifts in total concentration levels associated with pronounced changes in protein consumption were not due to a change in all of the proteins of the plasma. They could be accounted for by change in albumin alone, an observation which, in general, is in consonance with the original work of Govaerts (12). In theory, this reduces the problem of the mechanism of the food protein effect to an inquiry into the reasons for change in only one of the components of the plasma protein complex. In practice, however, we have not been able to take advantage of this simplification because we could not satisfy ourselves as to the reliability of our serum albumin measurements, and thus have had to be content with total protein determinations.

In experiments which we shall publish later, we have found that serum albumins given parenterally to rats are digested. Part of the injected albumin is used for anabolic purposes and part of its nitrogen is excreted as urea. This circumstance makes it worth while to study the time relations between the change in serum protein concentration and the change in protein metabolism that occur when the protein content of the diet is suddenly reduced to very low levels. If they occur together we shall have some reason to suspect that the serum albumin shift may be one of a series of interrelated events that accompany any pronounced reorganization in the relative magnitude of the rates of protein anabolism and catabolism.

We therefore studied the time relation between the decrease in protein metabolism that follows a change from a high to a low protein diet and the decrease in serum protein concentration which we quite confidently expected would follow the diet change. Four medical students were asked to make a sudden and pronounced alteration in their protein consumption. On Monday

and Tuesday they ate weighed diets that contained 2.5 gm. of protein per kilo body weight. On Wednesday and on Thursday up to 12 noon they were given only 10 gm. of protein a day with adequate calories. For their body weight this approximated a consumption of 0.13 gm. protein per kilo. Throughout Tuesday and Wednesday, and during the forenoon of Thursday, they collected urine at 5 hourly intervals during the day, between 7 a.m. and 10 p.m., with night collections between 10 p.m. and 7 a.m. In each individual the serum protein concentration was determined by the biuret method and the average results were confirmed by gravimetric determinations on pooled sera. In Table V the average results are given.

TABLE V

Failure of Any Significant Change in Serum Protein Concentration to Occur during the First 29 Hours after Change from a High to an Inadequate Protein Consumption. Averages from Four Subjects

Day	Protein consumption	Serum protein concentration			
		7:15 a.m.	11:45 a.m.	4:45 p.m.	9:45 p.m.
	gm. per kg.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Tuesday	2.5	8.45	8.23	8.65	8.42
Wednesday	0.1	8.37	8.49	8.75	8.74
Thursday	0.1	8.50	8.77		

It is apparent that there was no decrease in concentration during the first 29 hours following a change from high to a very low protein consumption. The hematocrit values also remained unaltered. Yet during this period the rate of urea excretion fell from 46.6 gm. per 24 hours on Tuesday, when they were on the high protein diet, to 17.5 gm. per 24 hours during the last collection on the inadequate protein ration. Over this time, also, the serum urea concentration declined from 49.2 to 23.1 mg. per 100 cc. Thus during the 29 hours in which the major part of the metabolic readjustments reflected by the excretion and concentration of urea was accomplished, the serum protein concentration did not fall.

Although the results given in Table V show that a great reduction in protein catabolism may occur without any fall in serum protein concentration, we still thought that we had only misjudged the time at which the fall occurred and we supposed we should observe it if we extended the period of observation. But when another group of four students took for 5 days a diet that was theoretically adequate in all respects except that it contained no more than traces of protein, we got the results given in Table VI.

The measurements given in Table VI were obtained by the biuret method in serum drawn every morning at 9:30 a.m. Instead of a fall in concentration

we find a steady daily rise over the whole 5 day period. This was accompanied by a slight increase in the hematocrit values. When the same subjects took diets containing 5, 10, or 15 gm. of protein there was no fall in serum protein concentration but rather, on the average, a slight and dubious increase without any definite change in packed cell volume over 5 or 6 day periods. Only when no protein at all was given were the results consistent and definite. Here the conjunction of an increase in both serum protein concentration and hematocrit reading made it seem possible that both phenomena might be a result of a decrease in total plasma volume. Dr. J. Hopper of the University of California Medical School was good enough to test this hypothesis for us. Comparing the results at the end of the period of zero protein consumption with those he obtained when the subjects were on an unrestricted diet, he found

TABLE VI

Increase in Serum Protein Concentration during the 5 Days Following Change from Unrestricted Food Consumption to a Diet That Contained No Protein

Subject	Days on a diet with no protein				
	1st	2nd	3rd	4th	5th
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
F.	7.48	7.63	7.68	8.29	8.71
G.	7.78	7.48	8.14	8.03	8.05
C.	6.17	6.83	7.08	7.55	8.31
L.	6.73	6.91	7.74	7.65	8.07
Averages.....	7.04	7.21	7.66	7.88	8.28

no consistent differences with the CO and the Evans' blue methods, and concluded that there was no evidence that the diet had had any effect on either total blood or plasma volume.

On all these diets there was a steady loss of body weight in spite of what appeared to be a more than sufficient consumption of calories. The readjustment of protein metabolism had been completed before the 5th day. On the zero protein diet the minimum urea excretion of 7.17 gm. per 24 hours came on the 3rd day and the serum urea concentration fell to 13.3 mg. per 100 cc. But the question with which we started, the relation in time between the change in protein metabolism and the expected fall in serum protein concentration, remains, of course, unanswered because instead of a fall we found a rise in concentration which was still continuing after all the metabolic change we can measure by urea excretion had been accomplished.

DISCUSSION

The subsidiary experiments, which were planned to throw light on the mechanism of the protein effect, have served only to show that the inverse

changes in serum protein concentration and packed cell volume which were our starting point, are not necessarily related as cause and effect. They have also demonstrated that we cannot derive from our results any general rule about the effect of change in protein consumption. The divergent direction of movement in protein concentration and hematocrit reading was found only in man. This phenomenon is further restricted to the particular conditions that obtain when the variations in the amounts of protein consumed are within the zone of what is defined as adequate for maintenance. When inadequate amounts are given, quite different or even contradictory effects are found. For this reason we have not been able to learn anything about the mechanism of the changes by pushing the dietary alterations to their extreme limit. From a negative point of view, however, it is of interest that a maximal and rapid decrease in protein metabolism may be associated with an increase in serum protein concentration not due to diminution of plasma volume. Nevertheless we must admit that the methods we used have been shown to be entirely inadequate for the purpose of answering the questions we have raised. Nor is it to be expected that they might have been solved if we had measured blood and plasma volumes and given the results in terms of total circulating plasma protein and red cell mass. Certainly much more would have been learned, for it is inadmissible to assume that blood volume remains constant when protein consumption is changed. In the work of Metcoff, Favour, and Stare (13) volume measurements were made but they were still not able to reach an unequivocal definition of the mechanisms involved in the total changes they measured. The excellently controlled and precise study of the effects of prolonged undernutrition in man reported by Keys and his collaborators (14) reveals how complex are the relations between the body as a whole and the volume and concentration changes in plasma protein and red cells, and to what a high degree any real understanding of the significance of these changes is dependent on a quantitative knowledge of total body composition in respect of the proportions of water in the cells, in the interstitial tissues, and in the circulating blood. The difficulty in reaching definitive conclusions about protein effects seems to lie in the circumstance that changes in protein consumption induce change in the system as a whole, as well as in all the parts that compose it. We thus have no fixed, unchanging, common reference value in terms of which we can express our measurements in order to compare them and learn their relative and absolute meaning in relation to the whole organism. At least we now know that body weight or any function of body weight does not give us what we need. Even if, admitting our incapacity to draw precise general inferences, we restrict our attention to the circulating blood whose volume we can at least approximately measure and in which relatively precise determinations of the concentration of its constituents can be made, we still do not have the information required for a full comprehension of the mechanism

of the effects of change in protein consumption. For this circulating blood is not a closed system, isolated from the body as a whole. In many respects it is like a reservoir into which newly formed protein molecules and red cells are always running, and out of which protein molecules and red cells as continuously are passing as they are metabolized or phagocytosed. What we need, in addition to the volume and concentration, are simultaneous measurements of the total inflow and total outflow of protein and red cells.

From an empirical point of view, however, it is of interest that in man a change from a low to a moderate, and then to a fairly high protein consumption, should be associated with an increase in specific gravity and a decrease in the packed cell volume. This protein effect is one of the minor factors which contribute to the total variability of these measurements in normal individuals.

SUMMARY

When the protein consumption of normal human individuals is increased from 0.5, to 1.5, to 2.5 gm. of protein per kilo body weight, the specific gravity of the plasma rises and the hematocrit value falls.

The analysis of variance demonstrates that the change in protein consumption is a significant but minor factor in determining the total variability of the observations.

When albino rats were given diets containing a small, a moderate, and a large amount of protein, there was an increase in serum protein concentration but no change in hematocrit value.

During the period over which the most rapid changes in rate of urea excretion and serum urea concentration occurred as normal human individuals passed from a 2.5 to an 0.1 gm. of protein per kilo body weight consumption, there was no change in serum protein concentration.

Over a 5 day period during which a diet that was adequate in calories but almost wholly devoid of protein was taken, the serum protein concentration of normal individuals steadily rose. This was associated with a slight increase in hematocrit value but no change in blood or plasma volume.

The protein effect is one of the minor factors that contribute to the variability of serum protein and hematocrit measurements in normal individuals.

The general conclusion is reached that we shall have to measure the rate at which red cells and protein enter and leave the circulating blood stream before we can hope to comprehend the mechanism of the protein effect.

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STUDIES
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Announcement

Volumes 131 and 132 of the *Studies from The Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

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The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1060 pages with 480 illustrations.

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THE ANTIBODY RESPONSE IN RABBITS TO KILLED SUSPENSIONS OF PATHOGENIC T. PALLIDUM

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Although reinfection with syphilis is not uncommon, it is usually observed in patients who have been treated early in the disease, and is observed only rarely in patients treated after the secondary lesions have spontaneously resolved (1). In syphilitic rabbits also, a solid immunity develops after a period of approximately 3 months (2). If such animals are cured by appropriate treatment and then reinoculated, even several million organisms usually fail to produce a second infection.

The mechanism of this immunity is not clear. It bears no obvious relationship to the presence of serum agglutinins or lysins; and opsonins have not been demonstrated. Syphilis does regularly cause the production of an antibody ("reagin") reactive with a ubiquitous lipoidal component of normal mammalian tissue. It is not yet clear, however, whether this reagin is actually a specific antibody to *T. pallidum* which happens to cross-react with a serologically related component of mammalian tissue (3), or whether, as suggested by Sachs, Klopstock, and Weil (4), syphilis causes the breakdown of host tissue and the release of tissue haptenes which are activated by spirochetal protein to form a complete antigen. In either case, the presence of so called Wassermann or flocculation reagin is apparently not the basis of the observed resistance to reinfection. In both rabbits and man, the titer of this antibody is highest in the early stages of the disease, long before there is a significant degree of immunity, and the titer may have dropped to negligible levels at a time when the immunity is maximal.

The only experimental evidence yet adduced for the presence in the serum of syphilitic animals or men of antibodies directed specifically against the causative organism is the demonstrations by Tani and his coworkers (5, 6) and by Turner (7) that when such serum is incubated with suspensions of *T. pallidum* and the mixtures injected into rabbits, the production of syphilitic lesions is then either delayed or prevented. In the latter instance, it is not yet known whether the admixture with serum actually prevents infection, or merely suppresses the appearance of the primary lesion, and makes the infection asymptomatic.

The present experiments were undertaken to determine the antibody response in rabbits to killed suspensions of pathogenic *T. pallidum*, administered in sa-

line suspension, or incorporated in water-in-oil emulsions as described by Freund (8). As will be here shown, the injection of even large numbers of the organisms, up to a maximum of 38 billion injected over a period of 13 weeks, did not produce demonstrable resistance to infection, since as few as ten organisms then sufficed to cause infection. The serum of such "immunized" animals had no demonstrable direct effect on the organisms *in vitro*.

The injection into rabbits of killed *T. pallidum* did, however, regularly cause the production in high titer of antibodies similar to those which develop in the course of syphilitic infection, the serum giving positive complement fixation (Wassermann) and flocculation tests with lipoidal extracts of normal tissue. The serum dilution titers reached a maximum level within 2 to 3 weeks. Thereafter, and despite continuing injection, the titers decreased progressively.

The present experiments therefore throw no light on the mechanism of immunity in syphilis. They do, however, constitute strong evidence that, as originally postulated by Wassermann, so called reagin may be simply an antibody to *T. pallidum*. Its anomalous reactivity with alcoholic extracts of normal mammalian tissue is probably to be attributed to the presence in such extracts of a lipid substance serologically related to one or more of the antigenic components of the organism (*cf.* (3)). Methods of preparing treponematal suspensions from rabbit chancres have not yet yielded suspensions sufficiently concentrated and sufficiently free from tissue components to warrant either their chemical fractionation or their use in absorption experiments.

EXPERIMENTAL

Antibody Response to the Injection of Saline Extracts of Testicular Chancres

Experiment 1. Intradermal Injection.—

Rabbit testicular chancres were removed at the height of the inflammatory reaction, minced with a scissors, and ground in a mortar with sand. The supernatant fluids, containing 10 million organisms per cc., were heated at 56°C. for 1 hour to kill the organisms, and were then kept frozen at −25°C. until used. Three rabbits were injected intradermally three times weekly, in amounts of 0.2 to 0.5 cc. One animal died after eight injections. The remaining two rabbits received 3.7 cc. (37 million organisms) over a 4-week period. Nine days after the last injection the serum Wassermann titers were 1:24 and 1:16. One of the two rabbits, inoculated intradermally with an unmeasured inoculum 7 days after the last injection, developed a darkfield positive lesion after a normal incubation period.

Experiment 2. Intradermal and Intraperitoneal Injection.—

A second group of four rabbits was injected both intradermally and intraperitoneally, the individual dose by the former route increasing from 0.2 to 0.5 cc., and by the latter route from 0.5 to 7 cc. In the course of twelve injections over a period of 4 weeks, the rabbits received a total of 3.7 cc. intradermally and 50 cc. intraperitoneally, and a grand total of 537,000,000 organisms. Nine days after the last injection, the serum Wassermann titers were 1:48, 1:32, 1:8, and 1:24. Three of the four rabbits inoculated at that time intradermally with an unmeasured inoculum developed typical darkfield positive lesions after a normal incubation period.

Experiment 3. Intravenous Injection.—

In a third experiment, begun in January, 1940, the organisms were injected intravenously and in much larger numbers than had previously proved feasible. Similarly prepared extracts of rabbit chancres were kept frozen at −25°C. until large amounts had been pooled. Two suspensions were prepared: one (325 cc. from a total of 132 testes) contained 75 million organisms and 5 mg. of N per cc.; the second suspension (235 cc. from 110 testes) contained 50 million organisms and 5.4 mg. of N per cc. Ten rabbits were injected intravenously three times weekly. Five died in the course of the injections. The remaining five received 35 cc. in

TABLE I

Antibody Response in Rabbits to Suspensions of T. pallidum in Testis Emulsion (Suspension Injected Intravenously Three Times Weekly)

Rabbit No.	No. of injections	Duration of immunization period	Total No. of organisms $\times 10^8$	Wassermann* titers on day No.			Flocculation* titers on day No.			Resistance to infection
				1	20	37	1	20	37	
		days								
42-87	13	37	4.4	0	6	32	0	16	48	Approximately 2×10^6 organisms were inoculated intradermally 2 days after the last immunizing injection. All the rabbits developed a darkfield positive lesion in 13 days, simultaneously with a group of non-immunized control animals.
42-88	13	37	4.4	0	18	48‡	0	48	96‡	
43-98	13	37	4.4	4	12	12	0	24	24	
44-40	13	37	4.4	0	<4	6	0	12	24	
44-49	13	37	4.4	2	<6	6	0	2	4	
43-22	13	37	4.4	0	4	—	0	3	—	
53-91§	12	33	4.8	0	—	½	0	—	12	Not done
53-92§	12	33	4.8	½	—	0	0	—	16	
53-97§	12	33	4.8	0	—	6	0	—	12	

* Wassermann and flocculation technics as described in (12), pages 1 and 5. Titers are expressed as the highest dilution of serum giving a positive result.

‡ This serum had no demonstrable agglutinating effect when added to an equal volume of a spirochetal suspension containing 10^8 organisms per cc., did not affect their motility, and the suspension remained infectious for rabbits (*cf.* Table II).

§ Injections by intravenous drip.

eight injections, and after a 10 day rest period were given a second course of 36 cc. in five injections. A total of 4.4 billion organisms was thus injected over a period of 35 days. The serum Wassermann and flocculation titers of this series of rabbits during the immunization period are summarized in Table I. As there shown, every animal injected developed a significantly increased reactivity with the alcoholic extracts of beef heart used as "antigen" in these tests.

This increased reactivity with mammalian tissue extractives presumably represents an antibody response to the organisms, although the necessary presence of tissue derivatives in the material used for injection complicates the inter-

pretation of the results (*cf.* page 381). It is true that similar extracts of normal testes, containing even larger amounts of solid and of N, did not cause the appearance of these antibodies in any one of fourteen rabbits tested. Further, such antibodies were not obtained when suspensions of cultured treponemata (Reiter strain) were added to the normal testis extracts in numbers corresponding to the number of pathogenic *T. pallidum* present in the chancre extracts. Antibodies reacting specifically with the saprophytic organism were, however, produced in high titer (9). These findings confirm the results obtained by many previous workers who have studied the antibody response to cultured spirochetes (*cf.* (4), page 292).

There was the further possibility that the treponematal suspensions used for immunization might have been infectious, despite the fact that they had been heated at 56°C. for 1 hour and stored at −25°C. for at least 2 weeks prior to use. This possibility was excluded by the demonstration that the popliteal lymph nodes of the immunized animals were not infectious for normal animals at the end of the immunization period.

Despite the appearance of a reagin-like substance in the serum in dilution titers comparable to those observed in the natural infection, when four of the animals of Table I were inoculated intradermally 2 days after the last immunizing injection, all developed typical darkfield positive lesions after a normal incubation period. Although the size of the inoculum was not accurately determined, it follows from the fact that many motile organisms were present in each microscopic field that on the order of 1 million to 10 million were injected in this challenge inoculation (10). This experiment must, therefore, be qualified by the fact that the inoculum used was many thousand times the minimal infectious dose (10), a complicating factor which was rectified in a following experiment.

Corresponding to the fact that the animals were not resistant to infection, when serum from rabbit 42-88, obtained at the end of the immunization period, was mixed with living organisms, there was no effect on either their motility or infectiousness (*cf.* Table II).

Experiment 4. Intravenous Drip.—

Three rabbits were injected, by slow intravenous drip, with a suspension containing 85 million organisms per cc. In thirteen such injections, given three times weekly over a period of 35 days, the animals received a total of 57 cc. of chancre extract, representing 4.8 billion organisms. The serologic response in those animals (bottom section of Table I), although not as pronounced as that observed in the preceding series, was nonetheless definite.

Antibody Response in Rabbits Immunized with a Concentrated Suspension of Sedimented T. pallidum

In later experiments concentrated suspensions of *T. pallidum* containing relatively small amounts of tissue extractives were prepared by differential centrifugation of the chancre emulsion.

Experiment 5.—

Testes were removed at the height of the inflammatory reaction 10 to 14 days after their inoculation, finely minced with scissors, and ground in a mortar and pestle with 0.85 per cent NaCl (approximately 5 cc. per testis). The mixture was lightly centrifuged to remove the tissue particles, and the sediment reextracted with a second portion of salt solution. The combined supernatant fluids were centrifuged in a conical head (International Centrifuge Co. No. 923) for 1 hour, at the end of which time from 75 to 90 per cent of the organisms had been sedimented as a whitish pellicle. The number of organisms in the sediment was estimated by counts on the suspension before and after centrifugation. The supernatant fluid was drained, the sediment was resuspended in salt solution to give a final concentration of 1 billion organisms per cc., and the suspension frozen at −25°C. Over a period of 2 months, nine such suspensions totalling 140 cc. were prepared from 192 testicular chancres. Some of these suspensions were killed before freezing by the addition of merthiolate to a 1:1000 concentration, and warming to 37°C. for 1 hour. The others were kept frozen until they were no longer infectious. No difference was noted between the two types of suspension, and they are not distinguished in the text.

TABLE II

The Failure of Serum from an "Immunized" Rabbit (42-88 of Table I) to Affect Either the Motility or Infectiousness of a Suspension of T. pallidum

"Immune" serum, with or without fresh guinea pig serum as complement, was added to 0.4 cc. of a suspension of *T. pallidum* containing 10^8 actively motile organisms per cc.

Tube No.	Normal serum	"Immune" serum, rabbit 42-88*	1:10 fresh guinea pig serum	Proportion of motile organisms after 4 hrs. in anaerobe jar at 37°C.	Infectiousness of mixture on intradermal inoculation of 0.2 cc. into normal rabbit
	cc.	cc.	cc.		
1	0.4	0	0	92	All the rabbits developed a darkfield positive lesion in 10 days
2	0	0.4	0	92	
3	0	0.4	0.4	90	
4	0	0	0.4	82	
5	0	0	0	74	

* Serum from rabbit 42-88 of Table I, drawn 37 days after beginning immunization with killed *T. pallidum*, when the serum Wassermann and flocculation dilution titers had reached 1:48 and 1:96, respectively.

In the first experiment with this material, thirteen rabbits were injected three times weekly to a total of sixteen injections in a period of 46 days. After the seventh injection, there was a rest period of 12 days before injections were resumed. The dosage per injection averaged 0.5 cc., or 500 million organisms, and the total number of treponemata injected during the immunization period was 7 billion.

As is indicated in Table III, every animal injected developed Wassermann and flocculation antibodies within a period of 16 days, the flocculation titers at this time varying from 1:16 to 1:64. No significant increase in this titer was observed on prolonged immunization. Instead, in most of the animals there was an indication that the serologic titer had reached a maximum in the first 2

weeks and thereafter decreased. (This was more clearly shown in the following experiment.) Three days after the last injection, a popliteal node was removed from the surviving animals, emulsified in 25 per cent serum, and injected into a normal rabbit to demonstrate that the suspensions used for immunization had actually been killed, and that the immunized animal had not been infected. None of the nodes proved infectious. The following day, and 4 days after the last immunizing injection, the animals were challenged either intradermally or intratesticularly with living inocula which varied from 100,000 down to 10 or-

TABLE III

The Antibody Response (Wassermann and Flocculation Tests) in Thirteen Rabbits Injected Intravenously with Sedimented Suspensions of T. pallidum

Sixteen injections totalling 7 billion organisms, over a period of 46 days: rest period of 12 days midway during immunization period.

Rabbit No.	Time after beginning of immunization, days						
	1	16	1	16	36	42	49
	Wassermann titer*		Flocculation titer*				
66-62	<2	6	0	16	12	8	12
66-87	<2	8	0	8	12	—	8
66-93	<2	12	0	16	12	Died	—
67-03	<2	12	0	16	Died	—	—
67-05	<2	24	½	64	16	24	32
67-22	<2	12	1½	16	24	24	8
67-25	<2	32	0	48	32	Died	—
67-28	<2	16	0	32	32	16	16
67-61	<2	—	0	6	16	8	8
67-70	<2	8	½	16	—	8	8
67-79	<2	6	½	8	12	6	8
67-80	<2	6	0	16	8	8	8
67-81	<2	24	0	32	24	8	—

* Technics as described in (12), pages 1 and 5.

ganisms. As is seen in Table IV, every animal inoculated intradermally, even those receiving as few as 10 organisms, developed a darkfield positive primary lesion after incubation periods which did not significantly differ from those in a control series of normal rabbits simultaneously inoculated. In the same rabbits injected intratesticularly, inocula of 100,000 and 1,000 organisms were regularly infectious, but two of three animals inoculated with 10 organisms did not develop demonstrable involvement of the testis within the period of observation (92 days). There was a suggestion also that the incubation period may have been prolonged in some of the animals which developed a darkfield positive lesion after intratesticular inoculation.

Experiment 6. Effects of Prolonged Immunization.—

In the infected rabbit, resistance to reinfection becomes manifest within a few weeks, but increases in degree progressively and slowly. From 3 to 6 months must pass before the animal becomes immune to reinfection with large inocula (2, 11). Accordingly, in a second experiment with sedimented organisms, an average of 1 billion killed organisms was injected three

TABLE IV

Incubation Period on Inoculation of Rabbits with living T. pallidum 4 Days after the Last Immunizing Injection

Rabbits of Table III

No. of organisms inoculated	Rabbit No. (*cf.* Table III)	Incubation period after	
		intradermal inoculation	intratesticular inoculation
		days	*days*
100,000	67-75	17	67
	67-70	13	42
	Normal controls simultaneously inoculated	9, 13, 13	21, 28, 35
1,000	66-62	21	28
	67-28	28	77
	67-61	21	35
	Controls	21, 21, 21	28, 28, 28
10	66-87	28	—*
	67-79	28	—*
	67-80	28	42
	Controls	28, 35	42, 42, 92

* No obvious testicular involvement for 3 months after inoculation, and testis darkfield negative at that time.

times weekly for a period of 4 months, and the total number of organisms injected was 38.5 billion.

As seen in Table V, and as is shown graphically in Fig. 1, the serologic antibody response previously noted was again observed. Maximum titers were again obtained within 2 weeks. Thereafter, however, the titers fell continuously, despite continuing injections at the same high level. Thus, the flocculation titer at the end of 2, 4, 6, 9, and 18 weeks averaged 28, 14, 6.5, 4.5, and 4.5, respectively. When 100 or 10 organisms were injected into two animals intradermally at the end of the immunization period, a syphilitic lesion developed within a normal incubation period (*cf.* Table V). As in the previous experi-

TABLE V

The Antibody Response in Rabbits Immunized for 4 Months with Suspensions of T. pallidum Sedimented from a Testicular Emulsion

Injections repeated three times weekly to a total of 40 injections and 38 billion organisms

Rabbit No.	1	Day No.					Resistance to infection with *T. pallidum*		
		14	28	42	63	126	Site of inoculation	No. of organisms injected	Result‡§
		Flocculation titer*							
70-08	1½	32	16	4	3	8	Testis	100	+37
70-88	1½	16	12	2	4	2			+72
71-06	0	32	12	1½	2	1		10	+37
71-39	1	32	8	4	2	2			∞§
70-30	½	16	8	1½	1	2			
71-38	0	32	16	4	2	8	Skin	100	+22
70-99	1	24	24	32	16	8			+22
71-07	0	16	16	2	1	1½		10	+33
70-67	0	16	8	4	4	—			+37
70-94	½	48	—	—	—	—	Conclusion: No demonstrable resistance to infection in 4 rabbits inoculated intradermally. Although 1 of 4 rabbits inoculated intratesticularly failed to develop a lesion, the significance of this fact is questionable (*cf.* text).		
70-97	1½	64	—	—	—	—			
71-09	1	32	—	—	—	—			
71-13	1	32	—	—	—	—			
71-14	0	16	—	—	—	—			
71-42	0	16	—	—	—	—			
Average flocculation titer..	0.5	28	14	6.5	4.5	4.5			

* Result of Eagle flocculation test, expressed as the highest dilution of serum giving a positive result.

‡ + = darkfield positive lesion developing in the inoculated area after the incubation period (days) indicated in the table. These results are to be compared with the following results in 4 normal rabbits simultaneously inoculated:

No. of organisms injected	Incubation period in control rabbits inoculated into	
	Skin	Testis
	days	*days*
100	37, 37, 33, 33	33, 29, 33, 37
10	27, 54, 37, ∞§	37, 37, 37

§ ∞ = No lesion at site of inoculation within period of observation. However, lymph nodes from rabbit 71-39 removed 110 days after inoculation proved infectious for normal rabbits, indicative of an asymptomatic infection.

ment, however, there was a suggestion of resistance in animals inoculated intratesticularly. Although two rabbits inoculated with 100 organisms both

developed a chancre, the incubation period was prolonged in one of the two. Of two other rabbits inoculated with 10 organisms, one failed to develop a lesion. A popliteal lymph node transferred to a normal animal 110 days after the original inoculation proved infectious, indicating that the animal had undergone an asymptomatic infection. Despite these suggestions of an altered response, it is nevertheless clear that no significant degree of immunity had developed in these animals under the impact of relatively large numbers of dead organisms.

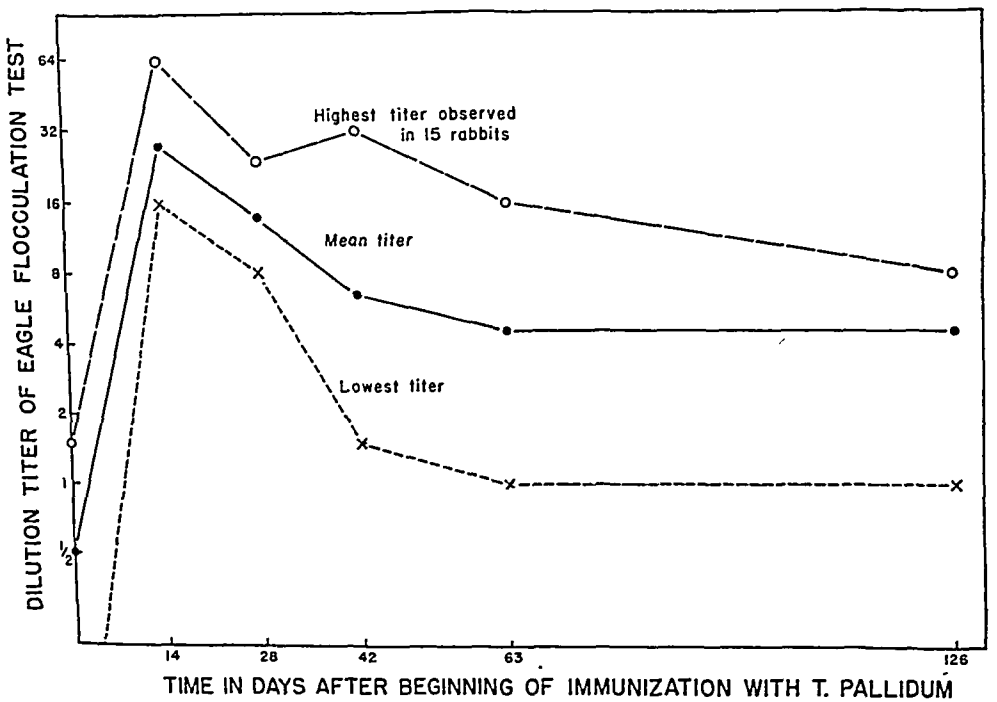

FIG. 1. The antibody response (Wassermann and flocculation reagin) in rabbits immunized by the intravenous injection of killed *T. pallidum* (after data of Table V).

As is indicated in Table VI, the serum from one of these rabbits, obtained midway in the immunization period, 63 days after the first injection, had only a questionable direct effect on pathogenic *T. pallidum*. The serum, either whole or diluted 1:10, was incubated with an equal volume of a chancre emulsion containing 10^8, 10^6, 10^4, or 10^2 organisms per cc., and 0.2 cc. of each mixture, representing inocula of 10^7, 10^5, 10^3, or 10 treponemata, was then injected into normal rabbits intradermally or intratesticularly. The three larger inocula caused the appearance of typical darkfield positive chancres within a normal incubation period. However, the suspension containing the smallest number of organisms proved non-infectious on intratesticular inoculation into two rabbits, this

despite the fact that one to two treponemata have been shown to be regularly infectious for rabbits on intratesticular inoculation (10). When the same suspension was similarly incubated with normal serum and inoculated, typical chancres developed in 28 to 35 days. The significance of this observation is nevertheless open to question. When 1:10 serum was used instead of whole serum, the "immune" and normal sera had the same effect; in each instance one of two rabbits inoculated developed a chancre.

TABLE VI

The Effect in Vitro of Serum from an "Immunized" Animal on the Virulence of T. pallidum

Serum from rabbit 70-08, obtained midway in immunization period, 63 days after the first injection (*cf.* Table V), was added to an equal volume of freshly prepared chancre extract diluted with 25 per cent rabbit serum to contain 10^8, 10^6, 10^4, and 10^2 treponemata per cc. After 1 hour at 37°C., 0.2 cc. of each mixture was injected both intradermally and intratesticularly into a normal rabbit. A fresh normal rabbit serum was simultaneously tested; and both the "immune" and normal serum were tested as both whole serum and in a 1:10 dilution.

No. of organisms inoculated after incubation with serum for 1 hr. at 37°C.		10^7		10^5		10^3		10	
Concentration of serum incubated with treponemata	Type of serum used	Incubation period on inoculation of mixture into							
		Skin	Testis	Skin	Testis	Skin	Testis	Skin	Testis
		days	*days*	*days*	*days*	*days*	*days*	*days*	*days*
Whole serum	Antiserum	14	Died	14	49	21	28	∞*	∞
	Normal serum control	14	21	14	21	21	35	28	35
1:10	Antiserum	14	43	21	21	21	35	∞	49
	Normal serum control	14	21	21	21	28	35	∞	49

* No lesion developed during period of observation (85 days).

Experiments 7 and 8. The Immunization of Rabbit with Killed T. pallidum Suspended in a Water-in-Oil Emulsion.—

Freund and his coworkers (8) and others (13, 14) have shown that the antigenic activity of bacteria is often considerably enhanced if they are suspended in the aqueous phase of a water-in-oil emulsion. This was produced by mixing an aqueous suspension of the organisms first in lanolin (or falba) and then emulsifying the mixture in *e.g.* mineral oil. They further showed that the incorporation of killed tubercle bacilli in the oil phase of such emulsions enhanced the sensitizing activity of the suspension.

A series of treponematal suspensions was therefore prepared in which extracts of rabbits' syphilitic testes, or the organisms sedimented from such extracts, were emulsified in anhydrous lanolin and then in mineral oil to form suspensions containing from 47 to 370 million organisms per cc. Rabbits were injected subcutaneously with varying amounts of these emulsions as indicated in the footnote to Table VII. At varying periods thereafter, specimens of blood were obtained by cardiac puncture, and their Wassermann and flocculation titers determined. In only one rabbit of the five (rabbit 60–66 injected with suspension E) was there a significant

TABLE VII

The Incubation Period of Syphilis in Five Rabbits Immunized with Killed T. pallidum, Suspended in a Water-in-Oil Emulsion

Suspension used for immunization*	1		2			3		
	Incubation period after inoculation with 100 organisms, 28 days after last injection		Incubation period on intradermal inoculation, 53 days after last injection, with			Results in intradermal inoculation with 2×10^6 organisms, 66 days after last injection		
						Live organisms		Dead organisms
	Skin	Testis	10^6 organisms	10^4 organisms	10^2 organisms	Incubation period	Size of lesion at that time	
						days	*min.*	
A	20	35	8	8	13	2	5 × 5	3 × 3 mm. in 24 hrs. 5 × 5 mm. in 48 hrs. 0 in 72 hrs.
B	17	45	8	14	13	2	Minute	0
C	14	39	23	20	14	7	3 × 6	0
D	20	48	8	14	14	2	5 × 5	0
E	17	27	14	20	14	7	3 × 5	0
Normal controls	>57‡	55				7	2 × 2	0
	27	48				7	Minute	0
	45	45				7	Minute	0
	38	39				10	5 × 5	0
	>55‡	38				7	Minute	0
Syphilitic controls			13	13	27	3	Minute	0
			10	13	23	7	3 × 7	0
			14	16	27	2§		

* One part of a treponematal suspension (either a direct extract of syphilitic testes, or the sedimented organisms from such an extract, resuspended in salt solution) was mixed with 1 to 1.5 parts of anhydrous lanolin, and the paste then stirred into 1 to 1.5 volumes of mineral oil. The composition of suspensions A to E listed was as follows:—

Suspension used for immunization	Final concentration of *T. pallidum*	Final concentration of tubercle bacilli	Total No. of treponemata injected × 10^6
	No. per cc. × 10^6	*mg./cc.*	
A	47	0	132
B	47	0.6	132 + tubercle bacilli
C	200	1.0	560 + tubercle bacilli
D	370	0	1040
E	370	1.75	1040 + tubercle bacilli

From 0.8 to 1 cc. of each suspension was injected subcutaneously three times at 6 day intervals.

‡ Animal died.

§ No lesion had developed in this time at the site of inoculation, but the expressed interstitial fluid contained actively motile organisms.

increase in antibody reactive with alcoholic tissue extracts, the Eagle flocculation titers on days 1, 6, 13, 23, 27, and 37 being 1½, 4, 4, 8, 12, and 8, respectively.

Forty-one days after the first immunizing injection, and 28 days after the last, the rabbits were inoculated intradermally and intratesticularly with 100 living organisms. Every immunized animal developed darkfield positive lesions. The incubation periods at the intratesticular sites did not differ significantly from those in control normal animals. There was, however, an indication that at the intradermal sites, the incubation period had perhaps been decreased. In the five immunized animals, the incubation period varied from 14 to 20 days, averaging 17; while in five control rabbits simultaneously inoculated, the incubation periods were 27, 38, and 45, and more than 55 days in the remaining two.

In order to retest this apparent increase in skin reactivity, 24 days after the first inoculation the same animals were reinoculated intradermally at three different sites with 10^6, 10^4, and 10^2 organisms. The incubation periods after these varying inocula are indicated in section 2 of Table VII. The incubation periods at the sites receiving large inocula did not differ significantly from those in control syphilitic rabbits. In the sites receiving a small inoculum, however, there was again a definite indication of a reduced incubation period, the observed period varying only between 13 and 14 days in the "immunized" animals as against 23 to 27 days in the control animals.

Further to test this apparent increase in sensitivity, 66 days after the last injection of the killed organisms, and 38 days after the first inoculation with living treponemata, fresh skin areas were injected with 2 million killed and live organisms. A third site in each rabbit was injected with a similarly prepared control extract of normal rabbit testes. Normal and syphilitic control animals were simultaneously injected. The results are summarized in section 3 of Table VII. The normal testis extract produced no reaction in any of the aninals. In one of the five immunized animals, but none of the controls, the killed organisms caused a significant erythematous reaction within 24 hours, which reached a peak in 48 hours, and had disappeared after 72 hours. The live organisms produced a darkfield positive lesion in 2 to 7 days, averaging 4, in the immunized animals, and in 2 to 10 days, averaging 6, in the control series.

In summary, the immunization of rabbits with killed *T. pallidum* suspended in water-in-oil emulsions after Freund, with or without the simultaneous injection of an adjuvant antigen (killed tubercle bacilli), did not cause the development of demonstrable resistance to infection with *T. pallidum*. There was, however, an indication that such immunization may perhaps have sensitized some of the rabbits to *T. pallidum*. The incubation period on intradermal inoculation with live organisms was decreased, and one of the five experimental rabbits developed an erythematous wheal 24 hours after the injection of a heat-killed suspension of the organisms.

DISCUSSION

Every one of thirty-seven rabbits injected intravenously with a total of 4.4 to 38 billion *T. pallidum* in aqueous suspension developed positive Wassermann or flocculation tests (complement fixation and precipitation with alcoholic extracts of beef heart) in significantly increased titer. The dilution titers of these tests in the normal serum controls varied from 0 to 1:4, averaging less than 1:2; the titers in the immunized series rose to as high as 1:96. This antibody response reached its maximum levels within 2 to 3 weeks, and thereafter either remained constant during the period of immunization or, in two experiments involving the continuing injection of large numbers of organisms, fell steadily in the course of the following 6 to 7 weeks.

The organisms used in these experiments were derived from rabbit testicular chancres, and the suspensions of necessity contained tissue extractives. The present experiments therefore do not constitute a rigorous demonstration that Wassermann and flocculation reagin is a specific antibody to *T. pallidum*. The possibility remains that the tissue extractives contain a haptene activated by the treponematal protein to form a complete antigen. However, there are two aspects of the present experiments which make that explanation unlikely. The first is the demonstration that control animals injected with extracts of normal testes, either with or without the addition of non-pathogenic Reiter spirochetes, failed to develop these antibodies. One would therefore have to assume either that pathogenic *T. pallidum* differs qualitatively from the cultivated organisms in its ability to activate the tissue haptene to a complete antigen, or that the syphilitic testes contained a haptenic substituent not present in normal tissue. The second point is the present demonstration that sedimented organisms containing relatively small amounts of tissue extractives were just as antigenic as the crude chancre emulsion from which they had been concentrated. The simplest explanation of the present data is that Wassermann or flocculation reactivity induced by the injection of these organisms, and presumably also the similar "reagin" elaborated during syphilitic infection, represent an antibody response to pathogenic *T. pallidum*. This was the thesis originally postulated by Wassermann when he developed the complement fixation test for syphilis which bears his name, and which was apparently negated by the subsequent demonstration that alcoholic extracts of normal mammalian tissue could be used as antigen. One need only assume that the treponemata and the mammalian tissues contain an immunologically related antigen. The final demonstration of that fact must await either the cultivation of the pathogenic organism, or the preparation of suspensions sufficiently concentrated or sufficiently free from tissue extractives to warrant their chemical fractionation or their use in cross-absorption experiments. Attempts in this direction are now in progress.

Paradoxically, the "immunized" rabbits in the present series did not regu-

larly develop a significant resistance to infection. Intradermal inoculation, in some experiments with as few as 10 treponemata, regularly resulted in a typical darkfield positive primary lesion at the site of inoculation, whether the animals had been immunized with totals of 30 million organisms intradermally, 537 million organisms subcutaneously, 4.4 to 38 billion organisms intravenously, or 130 to 1040 million organisms in a water-in-oil emulsion, administered over periods which varied from 13 days to 4 months. Recently, Magnuson, Halbert, and Rosenau (15) have also reported failure to produce a significant measure of resistance to infection by the injection of pathogenic *T. pallidum* suspended in oil-in-water emulsions of the type here used.

Three of five immunized rabbits which were challenged by the intratesticular inoculation of ten organisms failed to develop a primary lesion, while every one of five simultaneously inoculated controls was infected. In the one such animal tested, there had been an asymptomatic infection, the organisms having disseminated without producing a primary lesion at the site of inoculation. The significance of this observation, in the light of the results after intradermal inoculation, is open to question. The at best small measure of resistance to infection in these artificially immunized rabbits contrasts sharply with the fact that in the course of an actual infection the animals develop a solid immunity, to the degree that after they have been cured, inocula of many million organisms fail to produce even an asymptomatic second infection. Equally paradoxical, and perhaps related to the foregoing, is the fact that the immunized animals, while developing antibodies to a non-specific antigen (alcoholic extract of beef heart), failed to develop antibodies directly active against the treponematal suspension itself. The organisms were not specifically agglutinated, the sera did not give specific complement fixation with the treponematal suspensions, and as few as ten living organisms incubated for 1 hour with a high titered (Wassermann and flocculation) serum from an immunized animal, retained their infectiousness on inoculation into a normal animal. It is possible that the rabbits had been overimmunized, and were in a "negative phase" at the time they were tested for resistance to infection, or at the time their sera were tested for direct antitreponematal reactivity. There is the further possibility that the surface of the organisms contains a relatively non-antigenic material, and that the most effective antigen is intracellular. This might explain the development of serum antibodies which cross-react in high titer with non-specific antigens, despite the absence of reactivity (specific agglutination, complement fixation, lysis, or protection) with intact *T. pallidum*. However, this would not explain the pronounced immunity which develops in the course of actual syphilitic infection, but not in rabbits immunized with killed organisms. The final, if unlikely, possibility is that in none of the animals was there an antibody response to the treponemata as such, and that the Wassermann reagin was an antibody response to the small amounts of tissue extractives present in the

treponematal suspension. Under ordinary circumstances, those rabbit extractives are non-antigenic for rabbits, even if injected simultaneously with cultured treponemata. The pathogenic organisms may nevertheless possess a unique ability to activate the homologous tissue haptene to a complete antigen.

Of particular interest is the fact that in one series of animals, injected with organisms suspended in a water-in-oil emulsion, there was a suggestion that some of the rabbits may have been sensitized to the treponemata by the preceding immunization. The incubation period on intradermal inoculation with small numbers of organisms was decreased, and one of five rabbits reacted to the intradermal injection of killed organisms. The possible relationship of this observation to the late manifestations of the disease, in which relatively small numbers of organisms produce a disproportionately large tissue reaction, is apparent.

SUMMARY

The intravenous injection into rabbits of suspensions of dead *T. pallidum* derived from rabbit testicular chancres regularly caused the appearance of Wassermann and flocculation antibodies in significantly increased titer. Control suspensions of cultured treponemes (Reiter strain) added to extracts of normal testes were ineffective. This suggests that the Wassermann and flocculation reagin elaborated during syphilitic infection may be an antibody to *T. pallidum* which happens to cross-react with alcoholic extracts of mammalian tissue.

The antisera did not cause the agglutination of suspensions of pathogenic *T. pallidum*, living or dead, did not give specific complement fixation with those suspensions, and did not usually cause the living treponemata to lose their infectiousness.

Animals immunized with such aqueous suspensions for as long as 4 months, or with organisms suspended in a water-in-oil emulsion, were not demonstrably resistant to infection. As few as ten living organisms inoculated intradermally into animals "immunized" with as many as 38 billion dead treponemata regularly produced typical darkfield positive infections; and two of five animals inoculated intratesticularly with ten organisms were also infected.

The contradiction involved in the production of antibodies cross-reacting with a non-specific antigen, and the non-appearance of specific antibodies against the organism used as antigen, is discussed in the text.

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THE INHIBITORY EFFECT OF POLYSACCHARIDE ON MUMPS VIRUS MULTIPLICATION

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That certain polysaccharides, either of bacterial or non-bacterial origin, can modify the course of infection with pneumonia virus of mice (PVM) and inhibit multiplication of the virus in the mouse lung was demonstrated recently by Horsfall and McCarty (1). Very small amounts of polysaccharide, indeed but a few micrograms, if given by the intranasal route, cause striking inhibitory effects when injected either some days before or after inoculation of the virus. Green and Woolley (2) have shown that the multiplication of influenza A virus (PR8) in the allantoic sac of the chick embryo is inhibited as a result of the injection of apple pectin. Relatively large amounts of pectin, 25 to 50 mg., if given intra-allantoically, were found to be effective when injected either ½ hour before or 1 hour after inoculation of the virus. In a preliminary communication Ginsberg, Goebel, and Horsfall (3) recently reported that the capsular polysaccharide of Friedländer bacillus type B inhibits the multiplication of mumps virus in the allantoic sac of the chick embryo.

There are only a few prior reports which deal with the effect of polysaccharides on viruses or infections induced by these agents. Armstrong (4) demonstrated that after the intranasal injection of certain bacteria mice were less susceptible to intranasal inoculation with either St. Louis encephalitis virus or influenza A virus. In retrospect, it appears possible that this effect may have been due to a polysaccharide because the active component was present in sterile culture filtrates and withstood heating at 74°C. for 45 minutes. Levine and Frisch (5) found that certain bacterial extracts are capable of inhibiting the activity of bacteriophage strains which have the capacity to cause lysis of the parent bacterium. Subsequently, Burnet (6) confirmed and extended these observations, and Gough and Burnet (7) showed the phage-inactivating substance present in bacterial extracts to be a polysaccharide. Johnson (8) described the inactivation of tobacco mosaic and several other plant viruses by extracts of *Aerobacter aerogenes* as well as a variety of other microorganisms. Takahashi (9) obtained from yeast extract a polysaccharide which has the capacity to inactivate tobacco mosaic virus.

Our present interest in the inhibition of viral multiplication by means of polysaccharides derives from the idea that information concerning substances which block multiplication may provide clues to the nature of the unknown

substances in protoplasm which are essential for the multiplication of viruses. When it was found that a polysaccharide which is strikingly effective in blocking the multiplication of PVM in the mouse lung (1) is also capable of inhibiting the multiplication of mumps virus in the chick embryo (3), a detailed study of the phenomenon was undertaken. The results of this investigation form the subject of this report and that which accompanies it (10).

In the present communication it will be shown that capsular polysaccharides derived from type-specific strains of Friedländer bacilli cause a decrease in the susceptibility of the chick embryo to infection with mumps virus and, even in very small amounts, are effective as inhibitors of multiplication of the virus in this species. It will also be demonstrated that inhibition of mumps virus multiplication results when the appropriate polysaccharide is given before, or as late as 4 days after, inoculation of virus; that polysaccharides injected either into the allantoic or yolk sac inhibit multiplication of virus in the allantoic sac. In contrast, it will be shown that the multiplication of influenza A and influenza B, as well as Newcastle disease viruses is not inhibited by the polysaccharide which is active with respect to mumps virus and PVM. In addition, evidence will be presented which indicates that the specific serological properties of active polysaccharides are probably dependent upon certain chemical groupings distinct from those which mediate inhibition of viral multiplication. Evidence will be presented to show that the virus *per se* is not demonstrably inactivated by polysaccharide.

Materials and Methods

Viruses.—The following viruses were employed: mumps, influenza A, influenza B, and Newcastle disease. Mumps virus (MV) was obtained from Dr. Karl Habel, The National Institute of Health, Bethesda, Maryland, who had adapted it to multiplication in the allantoic sac of the chick embryo. As routine, the virus was cultivated in the allantoic sac of 7 to 9 day old embryos which after inoculation were incubated at 35°C. for 6 days. Thereafter, the infected embryos were chilled at 4°C. overnight, and the allantoic fluid removed.

The PR8 strain (11) of influenza A virus (IAV) and the Lee strain (12) of influenza B virus (IBV) were used. Both these strains have been passed many times through chick embryos and mice. Newcastle disease virus (NDV) was obtained from Dr. F. R. Beaudette, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey. Each of these 3 viruses was cultivated in the allantoic sac of 9 to 11 day old embryos which after inoculation were incubated at 35°C. for 48 hours. Infected allantoic fluids were harvested after preliminary chilling, as with mumps virus. Between experiments each of the viruses employed was stored in a solid carbon dioxide cabinet at −70°C. (13). It was found that the addition of nine parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) to one part of infected allantoic fluid protected virus infectivity, and permitted storage of each of the agents employed at −70°C. in cellulose nitrate tubes for long periods without loss of titer.

Chick Embryos.—Fertile White Leghorn eggs were incubated at 39°C. for 7 to 11 days before inoculation depending upon the virus employed. All embryos used in a given experiment were of the same age, and the eggs containing them were received in the laboratory on the same date.

Virus Infectivity Titrations.—Serial tenfold dilutions of infected allantoic fluid were made in sterile broth containing 10 per cent normal horse serum. A volume of 0.1 cc. of the desired dilution of virus was inoculated through a small drill hole in the egg shell directly over the allantoic sac in a region where the blood vessels could be avoided. At least 4 embryos were inoculated with each virus dilution and a minimum of 16 embryos was employed in each titration. After inoculation embryos were incubated at 35°C. In the case of mumps virus the period of incubation was 6 days; with influenza A, influenza B, and Newcastle disease viruses this period was 2 days. In all instances allantoic fluid was withdrawn after chilling of the eggs. Each allantoic fluid was tested for its capacity to cause agglutination of chicken erythrocytes. Virus titration end points (E.I.50) were calculated by the 50 per cent end point method of Reed and Muench (14).

Virus Hemagglutination Titrations.—Serial twofold dilutions of infected allantoic fluids were made in 0.85 per cent NaCl solution buffered at pH 7.2 (0.025 M phosphate). To 0.4 cc. of each dilution was added 0.4 cc. of a 1.0 per cent suspension of washed chicken erythrocytes. Readings were made after the tubes had stood 1 hour at room temperature. The end point was taken as the highest dilution at which definite (2+) agglutination of the red blood cells occurred.

Polysaccharide Preparations.—The following bacterial polysaccharides were employed: (1) capsular polysaccharides of Friedländer bacillus type A (Fr.A), type B (Fr.B), and type C (Fr. C), respectively (15, 16); (2) capsular polysaccharide of pneumococcus type II; (3) capsular polysaccharide of pneumococcus type III; (4) capsular polysaccharide of streptococcus MG (17); (5) levan synthesized from sucrose *in vitro* by the action of cell-free enzyme derived from *Streptococcus salivarius* type II (18); (6) dextran synthesized from sucrose *in vitro* by the action of cell-free enzyme derived from *Leuconostoc mesenteroides* (19). The following polysaccharide preparations of non-bacterial origin also were used: (1) blood group A substance; (2) apple pectin; (3) commercial corn starch. Before injection in the chick embryo each polysaccharide was dissolved in saline and heated at 70°C. for 30 minutes. In addition, the capsular polysaccharide of Friedländer bacillus type B was autoclaved during the course of preparation at 15 pounds' pressure for 20 minutes.

Immune Serum.—Rabbits were hyperimmunized by repeated intravenous injection of formalinized Friedländer bacillus type B vaccine. Immune serum was obtained 8 days after the last injection.

Precipitin Tests.—The presence of the capsular polysaccharide of Friedländer bacillus type B (Fr.B) was determined by the capillary precipitin technique (20) with specific immune rabbit serum. This procedure is of sufficient sensitivity to detect the polysaccharide in a concentration of the order of 1 μg. per cc. In order to determine accurately the extent of the serological activity of various solutions containing the degradation products of Fr.B, a turbidimetric measurement of the quantity of precipitate developed upon reaction with immune rabbit serum was employed (21).

EXPERIMENTAL

I. Reproducibility of Hemagglutination Titration End Points with Mumps Virus.—Levens and Enders (22) demonstrated that mumps virus causes agglutination of chicken erythrocytes. As is shown below, the hemagglutination technique can be used to measure the concentration of virus in infected allantoic fluid. In order to assess accurately the significance of differences in hemagglutination titers in various experiments, it was necessary to determine the reproducibility of such titers under relatively constant conditions. Individual

pools of allantoic fluids infected with mumps virus (MV), stored at −70°C. as described above, gave relatively constant virus infectivity titration end points (E.I.50) in the chick embryo. With different preparations the E.I.50 ranged between 10^{-6} and 10^{-7}. The hemagglutination titration end points obtained with allantoic fluids from 33 groups of 4 chick embryos inoculated intra-allantoically with 10^2 embryo infectious doses (E.I.D.) of mumps virus were tabulated, and the reproducibility of the end point was determined in the usual manner (23). In Table I the essential data and the results of the appropriate computations are presented. It should be pointed out that dilutions of 3 different pools of infected allantoic fluid were employed as the inocula in these experiments. The observed variation in end points obtained with each pool used as inoculum was considerably smaller than that noted when all end

TABLE I

Reproducibility of Hemagglutination Titration End Points with Allantoic Fluids from Chick Embryos Infected with Mumps Virus

Preparations of inocula employed	No. of groups of embryos* inoculated with 100 E.I.D.‡ intra-allantoically	Hemagglutination titer of allantoic fluids§ Log			Deviation from geometric mean Log			Standard deviation
No.		Lowest	Highest	Geometric mean	Least	Greatest	Mean	σ
3	33	−1.20 (1:16)	−2.76 (1:576)	−2.12 (1:132)	+0.01	−0.92	±0.26 (1.8-fold)	0.342 (2.2-fold)

* 4 chick embryos per group.

‡ E.I.D = embryo infectious doses.

§ Allantoic fluids obtained 6 days after inoculation; infected embryos were incubated at 35°C.

points obtained with the 3 inocula were considered. It will be noted that the mean deviation of the hemagglutination titration end points for the entire series is log ±0.26, and that the standard deviation of the distribution of the end points is 0.342. It can be shown that with any two individual end points, determined in the manner described above, a difference of 0.97 log unit should occur by chance only once in 20 times. In a single experiment the probability that a difference of this magnitude between two end points is significant is much greater. Throughout this paper a difference of 1.0 log unit between end points obtained with 2 groups of 4 embryos each will be considered as significant.

II. Hemagglutination as a Measure of Mumps Virus Concentration.—Habel (24) showed that mumps virus is infectious for the chick embryo, and can be cultivated in the allantoic sac as well as in the amniotic or yolk sacs. Beveridge and Lind (25) found that the hemagglutination titer of allantoic and

amniotic fluids infected with mumps virus was directly related to the complement-fixing titer of such fluids, and concluded that the hemagglutination titer was a function of virus concentration. However, Henle *et al.* (26) have shown that embryos infected with mumps virus elaborate a soluble antigen distinct from the virus *per se*, which, like the virus, is capable of fixing complement in the presence of specific antibodies. In the light of these findings it seemed necessary to determine by as direct means as possible the relationship between hemagglutination and virus titers. This problem was investigated in two different ways: (1) the rate of multiplication of the virus and the rate of increase in hemagglutination titer were determined and correlated; and (2) the effect of high gravitational fields upon both the virus titer and the hemagglutination titer was measured.

The rate of multiplication of mumps virus in the allantoic sac was studied in the following manner: In each experiment a number of chick embryos, 8 or 9 days of age, were inoculated intra-allantoically with 0.1 cc. of a 10^{-3} dilution of mumps virus (10^{3} E.I.D.). Each day thereafter allantoic fluid was removed from at least 4 embryos which then were discarded. The hemagglutination titer of each fluid was determined. In certain experiments pools were prepared containing equal volumes of allantoic fluid from each of a given group of embryos. Both the infectivity and hemagglutination titers of such pools were determined as described above.

The results of these experiments are presented graphically in Fig. 1 in which both the hemagglutination and infectivity titers of the allantoic fluid are plotted against time after inoculation with mumps virus. In Fig. 1 *B* the hemagglutination titers obtained in four separate experiments are shown. It is evident that during the first 3 days after inoculation the presence of virus was not demonstrable by means of the hemagglutination technique. After the 3rd day there was a rapid increase in titer which reached maximal levels on the 6th day. In Fig. 1 *A* both the infectivity and the hemagglutination titers obtained simultaneously in one experiment are shown. It will be seen that the rate of increase in the concentration of virus as determined by infectivity titrations paralleled the rate of increase in hemagglutination titer. When the experimental error of both titration techniques is taken into account, it can be shown that there is no significant difference between the two curves shown in Fig. 1 *A*. It appears from these results that virus and hemagglutination titers increase with time after inoculation of mumps virus into the allantoic sac in closely similar manners. Moreover, it appears that virus titers (E.I.50) of the order of $10^{-4.20}$ or higher are necessary before hemagglutination is demonstrable with mumps virus. Additional evidence on this point is presented below (*cf.* Tables II and VI).

The effect of high gravitational fields upon both the virus and hemagglutination titers of infected allantoic fluid was determined in the following manner: Allantoic fluids from infected

embryos were pooled, and the virus infectivity titer was immediately determined. The pooled fluid was then centrifuged at 15,000 R.P.M. for 30 minutes in a high speed vacuum apparatus (27). The centrifuge head was similar to that previously described (28) but was almost twice as large; the diameter = 30.1 cm. After centrifugation the supernate was decanted carefully and the sediment resuspended in a quantity of broth equal to the original volume. Virus infectivity titrations were then carried out with the supernate and the resuspended sediment

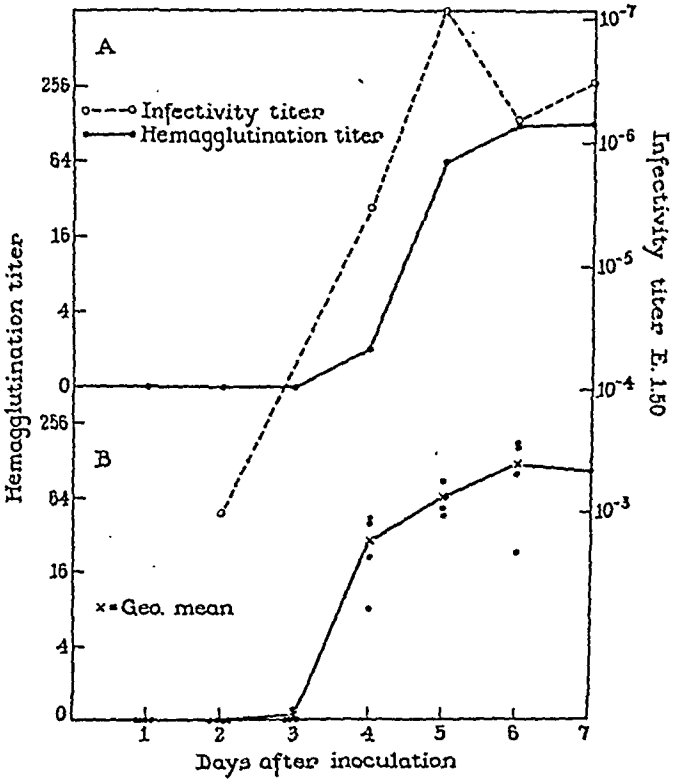

FIG. 1 *A*. Comparison of infectivity and hemagglutination titers of pools of allantoic fluids obtained from chick embryos in a single experiment at intervals after inoculation with mumps virus. Groups of 4 embryos were given 1,000 E.I.D. intra-allantoically.

FIG. 1 *B*. Hemagglutination titers of allantoic fluids obtained from chick embryos at intervals after intra-allantoic inoculation with 1,000 E.I.D. of mumps virus. Each end point represents the mean titer of at least 4 allantoic fluids.

Hemagglutination titrations, in duplicate, were performed with the uncentrifuged pool, supernate, and resuspended sediment.

The results of this experiment are shown in Table II. Both the virus and hemagglutination titers of the supernate were markedly lower than the corresponding titers obtained with the uncentrifuged pool. Conversely, both titers of the resuspended sediment were closely similar to those of the uncentrifuged pool. These results indicate that within the limits of the experimental techniques employed the effect of high gravitational fields upon both virus

and hemagglutination titers was similar if not identical. Because sedimentation of the virus was accompanied by proportional sedimentation of the component responsible for hemagglutination, it is evident that the two were not separable by gravitational force under the conditions of the experiment. This indicates that the property of infectiousness and the capacity to cause hemagglutination are associated with particles which, if not identical, are closely

TABLE II

Effect of High Speed Centrifugation on Infectivity and Hemagglutination Titers of Mumps Virus

Mumps virus		Infectivity titration		
Pooled allantoic fluids	Hemagglutination titer*	Dilution of material inoculated intra-allantoically	Infectivity score‡	Virus titration end point E.I.50§ Log
Before centrifugation	512	10^{-5}	4/4	
		10^{-6}	4/4	
		10^{-7}	3/4	−7.33
		10^{-8}	0/4	
Supernate after centrifugation at 15,000 R.P.M. for 30 min.	4	10^{-2}	4/4	
		10^{-3}	3/3	
		10^{-4}	4/4	−4.67
		10^{-5}	1/4	
		10^{-6}	0/4	
Sediment resuspended in original volume	512	10^{-4}	4/4	
		10^{-5}	4/4	
		10^{-6}	3/4	−6.50
		10^{-7}	1/4	
		10^{-8}	0/4	

* Expressed as the reciprocal.

‡ Numerator = number of allantoic fluids which caused hemagglutination. Denominator = number chick embryos inoculated.

§ E.I.50 = 50 per cent embryo infectivity end point.

similar in size and density. These results, as well as those dealing with the rate of increase in virus and hemagglutination titers, strongly suggest that mumps virus hemagglutination is caused by the virus particle itself and that the hemagglutination technique may indeed be used to measure virus concentration. It will be recalled that similar evidence has been obtained with influenza virus (29) and PVM (30).

In several experiments amniotic fluids, as well as 10 per cent suspensions of ground embryos which had been inoculated intra-allantoically, were tested for hemagglutination. In every instance virus could not be demonstrated by the

hemagglutination technique despite the fact that high titers were obtained with the allantoic fluids. Moreover, a virus infectivity titration carried out with an amniotic fluid pool yielded a titer of only $10^{-3.00}$. These results suggest that the chief site of multiplication of mumps virus after inoculation into the allantoic sac is the allantoic membrane.

III. Effect of Capsular Polysaccharide of Friedländer Bacillus Type B on the Susceptibility of Chick Embryos to Infection with Mumps Virus.—The results of preliminary experiments (3) indicated that the injection of capsular polysaccharide of Friedländer bacillus type B (Fr.B) caused inhibition of multiplication of mumps virus when as much as 10^4 E.I.D. was inoculated in the allantoic sac. Experiments were carried out to determine the extent to which the injection of Fr.B modified the susceptibility of the chick embryo to infection with mumps virus.

Each of a number of embryos was injected intra-allantoically with 1 mg. of Fr.B dissolved in 0.1 cc. of saline. Other embryos, which served as controls, were given 0.1 cc. of saline by the same route. After an interval of 3 hours groups of 4 embryos each were inoculated intra-allantoically with mumps virus in dilutions from 10^{-3} to 10^{-7}. In certain experiments the order of the injections was reversed and dilutions of virus were given 3 hours before the polysaccharide. Allantoic fluid was obtained from each embryo 6 days after inoculation and the hemagglutination titer determined. The virus titration end point was calculated as described above.

For purposes of clarity the results of one such experiment are presented in detail in Table III. It will be noted that, even though mumps virus was inoculated into the allantoic sac 3 hours before polysaccharide was given, the virus titration end point ($10^{-3.66}$) was strikingly lower than that obtained in control embryos ($10^{-6.33}$). It is also evident that the mean hemagglutination titer of allantoic fluids from each group of embryos which had received Fr.B polysaccharide was significantly lower than that of comparable controls. In many instances fluids from the former embryos failed to cause demonstrable hemagglutination. That the low hemagglutination titers observed were not attributable merely to the presence of polysaccharide in the allantoic fluids is evident from the findings presented in the accompanying paper (10); concentrations of Fr.B 10 times greater than those employed in this study did not lower the hemagglutination titer of mumps virus even when mixtures were held at 35°C. for as long as 48 hours.

In Table IV the results of such experiments are presented in summary form. In each instance the virus titration end point was definitely lower when determined in embryos which had been injected with Fr.B. The geometric mean of the differences between the titration end points obtained in control embryos and in those which were given polysaccharide is log -2.00. This indicates that 100 times more virus was required to initiate infection in Fr.B-treated embryos than in controls; that the susceptibility of such embryos to infection

with mumps virus was 100 times less than that of the controls. It is of interest to note that the effect of polysaccharide on susceptibility was equally as striking whether it was injected 3 hours before or after inoculation of virus.

The results of hemagglutination titrations carried out on allantoic fluids obtained from control and polysaccharide-treated embryos inoculated with varying amounts of mumps virus are shown in Table V. When amounts of virus from 10 to 10^4 E.I.D. were inoculated into embryos which had been given 1.0 mg. of Fr.B 3 hours previously, the hemagglutination titers obtained were in each instance significantly lower than those found with comparable control embryos. Only when as much as 10^5 E.I.D. of virus was given were similar titers obtained with allantoic fluids from polysaccharide-treated and control

TABLE III

Results of a Titration of Mumps Virus in Chick Embryos Injected with the Capsular Polysaccharide of Friedländer Bacillus Type B

1st injection 0.1 cc. intra-allantoic	Interval	2nd injection 0.1 cc. intra-allantoic	Incubation at 35°C.	Hemagglutination titer*					Infectivity score	Virus titration end point E.I.50 Log
				Individual allantoic fluids				Mean		
	hrs.		*days*							
MV 10^{-4}	3	Saline	6	64	512	8	32	154	4/4	
" 10^{-5}	"	"	"	64	32	512	8	154	4/4	
" 10^{-6}	"	"	"	16	128	16	0	40	3/4	−6.33
" 10^{-7}	"	"	"	0	0	0	0	0	0/4	
" 10^{-3}	"	Fr.B 1.0 mg.‡	"	0	16	4	8	7	3/4	
" 10^{-4}	"	" " "	"	0	4	0	0	1	1/4	−3.66
" 10^{-5}	"	" " "	"	0	0	8	0	2	1/4	

* Expressed as the reciprocal.

‡ Each embryo was given 1.0 mg. of polysaccharide intra-allantoically.

embryos. In view of the results obtained in the experiments described above, it appears that embryos given Fr.B are not only less susceptible to infection with but also are less capable of supporting multiplication of mumps virus than are control embryos.

IV. Toxicity of Fr.B Polysaccharide.—Embryos which were given Fr.B polysaccharide intra-allantoically showed no gross evidence of deleterious effects. They grew and developed at the same rate as controls and died no more frequently than embryos given saline by the same route. This was true even when 10 mg. of Fr.B per embryo was injected which is 5 times the quantity used in any experiment. The chorioallantoic membranes of 8 day embryos injected with 1 mg. of Fr.B were examined microscopically after the embryos had been incubated for either 2 or 6 days. The membranes were fixed in

Zenker's solution and stained with hematoxylin and eosin. There were no demonstrable microscopic lesions in any of the embryonic tissues examined. It appears, therefore, that Fr.B polysaccharide in the quantities given is innocuous for and has no evident toxic effects upon the chick embryo.

V. Effect of Fr.B Polysaccharide on Virus Titer after Injection with Mumps Virus.—It was necessary to determine directly whether or not allantoic fluid

TABLE IV

Titrations of Mumps Virus in Chick Embryos Injected with the Capsular Polysaccharide of Friedländer Bacillus Type B

1st injection 0.1 cc. intra-allantoic	Interval	2nd Injection 0.1 cc. intra-allantoic	Incubation at 35°C.	Virus titration end point E.I.50 Log	Difference from control Log
	hrs.		*days*		
Saline	3	MV dilutions*	6	−6.17	−2.17
Fr.B‡	"	" "	"	−4.00	
Saline	"	" "	"	−5.63	−1.63
Fr.B‡	"	" "	"	−4.00	
Saline	"	" "	"	−6.17	−1.67
Fr.B‡	"	" "	"	−4.50	
Saline	"	" "	"	−6.60	−1.85
Fr.B‡	"	" "	"	−4.75	
MV dilutions*	"	Saline	"	−6.33	−2.67
" "	"	Fr.B‡	"	−3.66	
Geometric mean		Saline		−6.18	−2.00
		Fr.B		−4.18	

* Serial tenfold dilutions from 10^{-3} to 10^{-7} were employed in each titration. A group of at least 4 embryos was inoculated with each dilution.

‡ 1.0 mg. of polysaccharide per embryo.

from embryos infected with mumps virus and also given Fr.B polysaccharide showed a reduction in virus infectivity titer which corresponded to the observed reduction in hemagglutination titer.

Four 9 day old embryos were inoculated intra-allantoically with 10^2 E.I.D. of mumps virus. Three hours later each of 2 of the embryos was given 1.0 mg. of Fr.B by the same route; the remaining 2 were given 0.1 cc. of saline. They were then incubated at 35°C. for 6 days after which allantoic fluids were removed and two pools prepared. The hemagglutination titer and, in addition, the virus infectivity titer of the pools were determined.

The results of this experiment are shown in Table VI. The allantoic fluid pool obtained from control embryos had a hemagglutination titer of 1:256 and a virus titer of $10^{-7.00}$, whereas that obtained from embryos which received Fr.B caused no hemagglutination and had a virus infectivity titer of only $10^{-4.25}$. It is evident that the differences between the hemagglutination titers (*i.e.*, log −2.41) and virus infectivity titers (*i.e.*, log −2.75), respectively, of the allantoic fluid pools were closely similar. It appears, therefore, that the actual concentration of mumps virus present in the allantoic fluid of polysaccharide-

TABLE V

Reduction in Hemagglutination Titer of Mumps Virus in the Allantoic Fluid of Chick Embryos Injected with the Capsular Polysaccharide of Friedländer Bacillus Type B

1st injection 0.1 cc. intra-allantoic	Interval	2nd injection Intra-allantoic mumps virus	Incubation at 35°C.	Hemagglutination titer of allantoic fluids* Log	Difference from controls Log
	hrs.	*E.I.D.*	*days*		
Saline	3	10	6	−2.01	−1.53
Fr.B‡	"	"	"	−0.48	
Saline	"	10^2	"	−2.27	−1.93
Fr.B‡	"	"	"	−0.34	
Saline	"	10^3	"	−2.16	−1.53
Fr.B‡	"	"	"	−0.63	
Saline	"	10^4	"	−2.25	−1.18
Fr.B‡	"	"	"	−1.07	
Saline	"	10^5	"	−2.48	−0.37
Fr.B‡	"	"	"	−2.11	

* Geometric mean of the hemagglutination titers of allantoic fluids from at least 2 groups of embryos (4 embryos per group).

‡ 1 mg./embryo.

treated embryos was markedly lower than that obtained with controls. Moreover, it appears that this difference can be measured with as much precision by means of the hemagglutination technique as by virus infectivity titrations.

VI. Effect of Fr.B Polysaccharide upon Infectivity of Mumps Virus.—It was of importance to determine whether the polysaccharide (Fr.B) employed in the preceding experiments caused inactivation of mumps virus *in vitro*. If this occurred, it appeared probable that an explanation for the effects observed *in vivo* might be obtained.

Allantoic fluid from embryos infected with mumps virus was diluted 10^{-1} in sterile normal horse serum. To one aliquot was added an equal volume of Fr.B in a concentration of 10

mg. per cc. in saline. To another aliquot an equal volume of saline was added. The mixtures were held at 4°C. for 30 minutes after which serial dilutions were prepared. Virus infectivity titrations were then carried out.

The results of this experiment are presented in Table VII. It will be noted that, despite the presence of a relatively high concentration of polysaccharide in the initial mixture, the infectivity titer of the virus following treatment with Fr.B was identical with that of the control. These findings indicate clearly that under the conditions of the experiment there was no demonstrable inactivation of virus in the presence of a large quantity of polysaccharide. Moreover, they suggest that the effects observed *in vivo* are not to be explained on the basis of direct action by Fr.B on mumps virus.

TABLE VI

Reduction of Both Hemagglutination and Infectivity Titers of Mumps Virus in the Allantoic Fluid of Chick Embryos Injected with the Capsular Polysaccharide of Friedländer Bacillus Type B

1st injection Intra-allantoic	Interval	2nd injection Intra-allantoic	Incubation at 35°C.	Hemagglutination titer of allantoic fluid	Infectivity titrations of allantoic fluid		
					Dilution	Infectivity score	Virus titration end point E.I.50 Log
E.I.D.	*hrs.*		*days*				
MV 10^2	3	Saline	6	1:256	10^{-5}	3/3	
					10^{-6}	3/4	
					10^{-7}	2/4	−7.00
					10^{-8}	1/3	
" "	"	Fr.B 1.0 mg./embryo	"	0	10^{-3}	3/3	
					10^{-4}	2/4	−4.25
					10^{-5}	1/3	
					10^{-6}	0/4	

VII. Quantity of Fr.B Polysaccharide Required to Inhibit Multiplication of Mumps Virus.—It appeared of interest to determine the smallest amount of Fr.B polysaccharide which could cause a significant reduction in the multiplication of mumps virus in the allantoic sac.

Approximately 100 E.I.D. of virus was used in these experiments and embryos were inoculated intra-allantoically with virus 3 hours before the polysaccharide was injected. A number of experiments were performed; in each instance two control groups of 4 embryos given 0.1 cc. of saline 3 hours after inoculation of virus were included. Allantoic fluids were removed 6 days after inoculation and their hemagglutination titers determined.

The results obtained are presented graphically in Fig. 2 in which the logarithm of the difference in the hemagglutination titers of polysaccharide-treated

and control embryos is plotted against the quantity of Fr.B injected. It will be noted that Fr.B polysaccharide was capable of inhibiting mumps virus multiplication to a significant degree when as little as 5 μg. per embryo was injected into the allantoic sac. When relatively large amounts of Fr.B (*i.e.*, 0.5 to 1.0 mg. per embryo) were given, the extent to which virus multiplication was inhibited was definitely greater than when smaller amounts were injected. However, the degree of inhibition obtained after the injection of 200 μg. was not strikingly different from that obtained when only 5 μg. per embryo was given. It will be recalled that in similar experiments with PVM it was found

TABLE VII

The Effect of the Capsular Polysaccharide of Friedländer Bacillus Type B on Mumps Virus in Vitro

Mixture held at 4°C. 30 min.		Dilution of mixture inoculated intra-allantoically	Constituents of inoculum		Hemagglutination titer of allantoic fluids*	Infectivity score	Virus titration end point E.I.50 Log
Mumps virus dilution	Diluent		Mumps virus	Fr.B			
				μg./embryo			
$10^{-1.3}$	Saline	10^{0}	$10^{-1.3}$	0	171	3/3	
		$10^{-2.7}$	10^{-4}	"	224	4/4	
		$10^{-3.7}$	10^{-5}	"	144	4/4	
		$10^{-4.7}$	10^{-6}	"	171	3/3	
		$10^{-5.7}$	10^{-7}	"	21	1/3	−6.75
$10^{-1.3}$	Fr.B 5 mg./cc.	10^{0}	$10^{-1.3}$	500	96	2/4	
		$10^{-2.7}$	10^{-4}	1.0	20	3/4	
		$10^{-3.7}$	10^{-5}	0.1	156	4/4	
		$10^{-4.7}$	10^{-6}	0.01	149	3/3	
		$10^{-5.7}$	10^{-7}	0.001	4	1/4	−6.67

* Expressed as the reciprocal of the mean of the hemagglutination titers obtained 6 days after inoculation.

that 1.6 μg. of Fr.B per mouse was capable of causing inhibition of virus multiplication (1).

VIII. Effect of the Time Interval between Injections of Virus and Polysaccharide.—Experiments were carried out to determine the duration of the interval after inoculation with mumps during which injection of Fr.B polysaccharide would cause inhibition of virus multiplication.

In these experiments embryos were inoculated intra-allantoically with 100 E.I.D. of mumps virus. Three hours before and after the inoculation of virus, as well as at intervals of 24 hours thereafter, each of 4 embryos was given a single injection of 1.0 mg. of Fr.B by the same route. The eggs were incubated at 35°C. for 6 days following inoculation with mumps virus, and the hemagglutination titer of each allantoic fluid was then determined.

The results obtained are presented in Fig. 3. The logarithm of the difference between the hemagglutination titers of the allantoic fluids obtained from polysaccharide-treated and control embryos is plotted against the time interval which elapsed between inoculation of virus and injection of Fr.B. It is important to point out that all allantoic fluids were obtained 6 days after inoculation with virus. It is evident that a single injection of 1.0 mg. of polysaccharide,

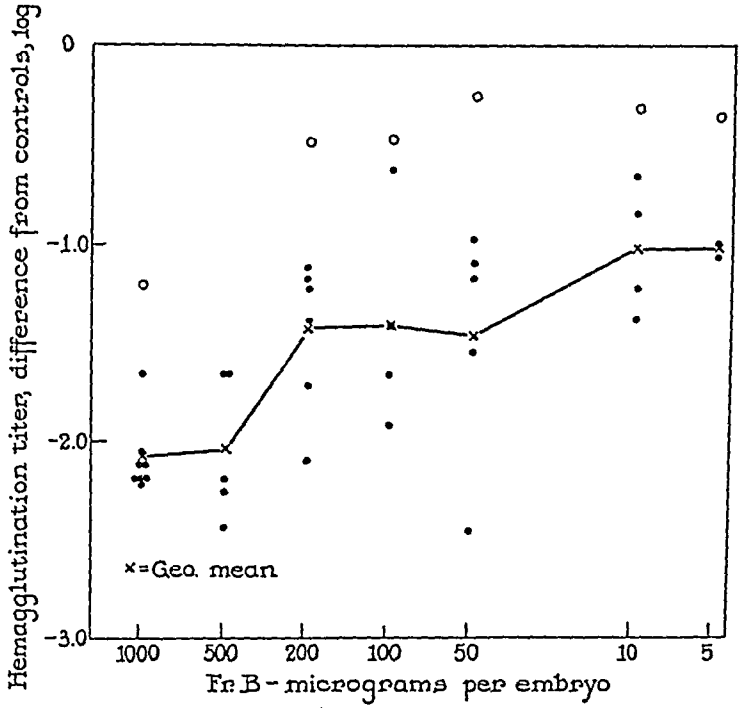

FIG. 2. Effect of different quantities of capsular polysaccharide of Friedländer bacillus type B (Fr.B) on the hemagglutination titer of allantoic fluids obtained from chick embryos inoculated with 100 E.I.D. of mumps virus. Polysaccharide was injected intra-allantoically 3 hours after virus. Groups of 4 embryos were employed; each end point represents the mean difference in titer between one experimental and two control groups. Results indicated by open circles were obtained in a single experiment and, because they deviate systematically from the other results, they were not included in the calculation of geometric mean.

whether given 3 hours before or as long as 48 hours after inoculation with mumps virus, markedly inhibited viral multiplication. Although inhibition became progressively less striking as the time interval was further increased, a significant degree of inhibition was demonstrated when the interval was as long as 96 hours, though not when it was 120 hours. For purposes of comparison the rate of multiplication of mumps virus in the allantoic sac of control embryos is also shown in Fig. 3. It will be seen that the effect of the polysaccharide was most marked when multiplication of virus had not yet reached the minimal threshold (*i.e.*, virus titer of $10^{-4.30}$) at which it can be demonstrated by means of the

hemagglutination technique. As further multiplication occurred with increasing time, the effect of the polysaccharide became less and less evident. When nearly maximal multiplication had taken place (*i.e.*, 5 days after inoculation), injection of polysaccharide caused no significant effect. It is of importance to emphasize that at no time following inoculation of the virus did injection of polysaccharide cause a *reduction* in the concentration of virus already present.

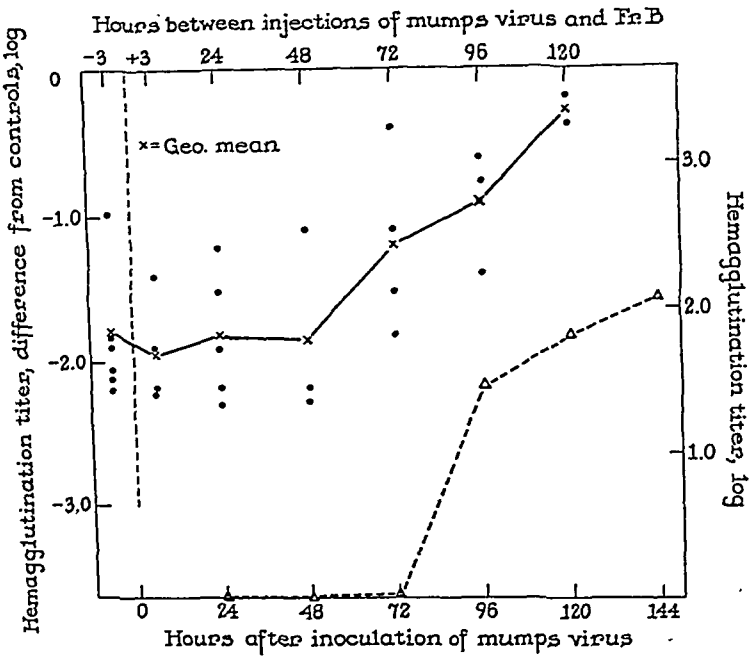

FIG. 3. Upper graph: The effect of time between inoculation with mumps virus and injection of Fr.B polysaccharide on the hemagglutination titer of allantoic fluids obtained 6 days after viral infection. Groups of 4 embryos were employed; each end point represents the mean difference in titer between one experimental and two control groups.

Lower graph: The rate of increase in the hemagglutination titer of the allantoic fluid of control embryos inoculated with mumps virus.

It appears that in each instance the effect observed is to be explained entirely on the basis of inhibition of further viral multiplication.

IX. Effect of Injection of Fr.B Polysaccharide into the Yolk Sac.—A number of experiments were carried out to determine whether injection of Fr.B polysaccharide by a route other than the allantoic would also cause inhibition of multiplication of mumps virus in the allantoic sac. It was found that among 45 embryos, each of which was given 5 mg. of polysaccharide into the yolk sac and then inoculated with 100 E.I.D. of virus intra-allantoically, 24 (53.2 per

cent) yielded allantoic fluids with significantly lower hemagglutination titers than were obtained in appropriate controls. When precipitin tests were performed with anti-Fr.B serum, it was found that the polysaccharide was present in the allantoic fluid of some embryos, but was not demonstrable in that from others despite the fact that all had been given the same amount of Fr.B in the yolk sac. It was found also that allantoic fluids in which Fr.B was demonstrable gave in almost every instance low hemagglutination titers, whereas those in which Fr.B was not demonstrable showed titers similar to those obtained with controls. That the low hemagglutination titers were not caused by a direct effect of Fr.B on the virus can be concluded from the finding that mixtures of polysaccharide and virus gave theoretical titers as is shown in the accompanying paper (10). It appears, therefore, that when polysaccharide was transported from the yolk sac to the allantoic sac, and appeared at the latter site in demonstrable amounts, inhibition of virus multiplication in the allantoic sac was obtained. Why polysaccharide failed to be so transported in approximately 50 per cent of embryos is not known.

X. Effect of Various Polysaccharides upon Multiplication of Mumps Virus.— It has been shown that a variety of polysaccharides are effective in inhibiting multiplication of PVM in the mouse lung (1). In view of the fact that Fr.B polysaccharide exhibited this property and, in addition, inhibited multiplication of mumps virus, it was of interest to study other carbohydrates which actively inhibit multiplication of PVM as well as others which show no such activity.

It will be seen from the results shown in Table VIII that, with the exception of Fr.B polysaccharide, none of the preparations tested which were effective in inhibiting multiplication of PVM in the mouse lung had a similar effect upon infection with mumps virus in the allantoic sac of the chick embryo. The capsular polysaccharide of streptococcus MG and blood group A substance inhibit multiplication of PVM (1), but were entirely ineffective when tested with mumps virus. Recent studies in this laboratory have shown that dextran synthesized from sucrose by means of cell-free enzyme obtained from *Leuconostoc mesenteroides*[1] was capable of inhibiting multiplication of PVM. However, this carbohydrate also was ineffective with mumps virus. It is of considerable interest that capsular polysaccharide of pneumococcus type II, although immunologically related to capsular polysaccharide of Friedländer bacillus type B, likewise had no significant effect on the multiplication of mumps virus. Moreover, type II capsular polysaccharide has been found to have no effect upon the multiplication of PVM.[1]

Because of their serological relationship, experiments were carried out to determine whether capsular polysaccharide of pneumococcus type II was ca-

[1] Unpublished observations.

pable of blocking the effect of Fr.B polysaccharide. Two mg. of type II capsular polysaccharide was injected into the allantoic sac of embryos 3 hours before

TABLE VIII

The Effect of Various Polysaccharides upon the Multiplication of Mumps Virus in the Allantoic Sac of the Chick Embryo

1st injection 0.1 cc. intra-allantoic		Interval	2nd injection Intra-allantoic mumps virus	Incubation at 35°C.	Hemagglutination titer* of allantoic fluids of embryos injected with		Difference from controls Log
Polysaccharide	Amount				Saline Log	Polysaccharide Log	
	mg./embryo	*hrs.*	*E.I.D.*	*days*			
Fr.B	1	3	10^2	6	−2.27	−0.34	−1.93
Pneumococcus type II SSS‡	1	"	10	"	−1.61	−1.38	−0.23
" "	2	"	10^2	"	−2.08	−1.43	−0.65
Pneumococcus type III SSS‡	1	"	10	"	−2.10	−1.36	−0.74
" "	1	"	10^2	"	−2.30	−1.57	−0.73
Apple pectin	1	"	10	"	−2.62	−2.48	−0.14
" "	1	"	10^2	"	−2.37	−2.98	+0.61
" "	2	"	10	"	−1.52	−2.05	+0.53
Streptococcus MG, SSS‡	1	"	10	"	−1.61	−1.66	+0.05
Blood group A	1	"	10	"	−1.61	−1.85	+0.24
Dextran§	1	"	10	"	−2.62	−2.48	−0.14
"	1	"	10^2	"	−2.37	−2.33	−0.04
Levan‖	1	"	10^2	"	−1.94	−1.90	−0.04
Corn starch	1	"	10^2	"	−1.79	−2.11	+0.32

* Expressed as the geometric mean.

‡ SSS = capsular polysaccharide.

§ Produced *in vitro* from sucrose by cell-free enzymes from *Leuconostoc mesenteroides*.

‖ " " " " " " " " " " " *Streptococcus salivarius* type II.

0.2 mg. of Fr.B was given; 100 E.I.D. of mumps virus was then inoculated 3 hours later. In each instance Fr.B polysaccharide inhibited the multiplication of mumps virus in undiminished degree. It appears, therefore, that type II

capsular polysaccharide does not act as an antagonist with respect to Fr.B. The striking differences noted in the biological activities of these two serologically related polysaccharides suggest that this function is not necessarily related to immunological similarity. In this connection it is of considerable interest that a small quantity of Fr.B polysaccharide neutralized with an amount of specific immune serum sufficient to provide an excess of antibody caused as marked inhibition of multiplication of mumps virus as did the same quantity

TABLE IX

Effect of the Capsular Polysaccharides of Friedländer Bacillus Types A, B, and C, Respectively, on Multiplication of Mumps Virus in the Chick Embryo

1st injection Intra-allantoic	Interval	2nd injection Intra-allantoic	Amount of polysaccharide	Hemagglutination titer of allantoic fluids* Log	Difference from controls Log
	hrs.		*mg./embryo*		
Fr.A‡	3	MV 10^2 E.I.D.	1.0	−0.90	−1.21
MV 10^2 E.I.D.	"	Fr.A‡	0.2	−0.70	−1.36
" " "	"	"	0.05	−1.23	−0.73
" " "	"	"	0.01	−0.70	−1.36
Fr.B‡	"	MV 10^2 E.I.D.	1.0	0	−2.11
MV 10^2 E.I.D.	"	Fr.B‡	0.2	−0.90	−1.16
" " "	"	"	0.05	−0.48	−1.58
" " "	"	"	0.01	−0.70	−1.36
Fr.C‡	"	MV 10^2 E.I.D.	1.0	0	−2.11
MV 10^2 E.I.D.	"	Fr.C‡	0.2	0	−2.06
" " "	"	"	0.05	0	−2.06
" " "	"	"	0.01	0	−2.06

* Geometric mean of the hemagglutination titers of allantoic fluids obtained 6 days after inoculation.

‡ Fr.A = capsular polysaccharide of type A Friedländer bacillus.
Fr.B = " " " " B " "
Fr.C = " " " " C " "

of Fr.B in the absence of serum. Chemical evidence bearing on this point is presented below.

The capsular polysaccharides of Friedländer bacillus types A and C, respectively, were also tested to determine whether they were capable of inhibiting the multiplication of mumps virus in the allantoic sac of the chick embryo. As is shown in Table IX, not only were both these polysaccharides strikingly effective as inhibitors of the multiplication of mumps virus, but also they significantly inhibited multiplication when quantities as small as 10 μg. were given. It is of interest that both Fr.A and Fr.C polysaccharides have been found to act as inhibitors with respect to PVM.[1]

XI. The Effect of Fr.B Polysaccharide on the Multiplication of Other Viruses.—The inhibitory effect of Fr.B polysaccharide upon viruses other than mumps also was investigated. In Table X the results of these experiments are shown. It will be observed that even when very small amounts of influenza A, influenza B, or Newcastle disease viruses were inoculated 3 hours after relatively large amounts of polysaccharide had been injected intra-allantoically, no evidence of inhibition of multiplication of any of these viruses was obtained. It appears evident that Fr.B polysaccharide, when used in amounts which are strikingly effective against mumps virus, was not capable of affecting significantly the

TABLE X

Effect of the Capsular Polysaccharide of Friedländer Bacillus Type B on the Multiplication of Influenza A, Influenza B, and Newcastle Disease Viruses in the Chick Embryo

Virus inoculated	No. E.I.D. injected intra-allantoically	Amount Fr.B	Incubation at 35°C.	Hemagglutination titer of allantoic fluids* of embryos injected with		Difference from control Log
				Saline‡ Log	Fr.B‡ Log	
		mg./embryo	*days*			
Influenza A	10	1.0	2	−2.53	−2.60	+0.07
" "	10^2	1.0	"	−2.85	−2.33	−0.52
" "	10	5.0	"	−2.53	−2.41	−0.12
Influenza B	10	0.6	"	−2.28	−1.88	−0.40
" "	10^2	0.6	"	−2.01	−1.86	−0.15
Newcastle disease	1	0.6	"	−1.90	−2.53	+0.63
" "	10	0.6	"	−2.58	−2.58	0
" "	10^2	0.6	"	−2.65	−2.58	−0.07

* Expressed as the mean of the hemagglutination titers.

‡ Saline or polysaccharide was injected intra-allantoically 3 hours before inoculation of virus.

hemagglutination titers of the allantoic fluids of embryos infected with influenza A, influenza B, or Newcastle disease viruses. Moreover, it was found that the infectivity titers of each of these viruses were closely similar both in polysaccharide-treated and in control embryos. Other experiments have demonstrated that Fr.B polysaccharide given intranasally does not lower the virus infectivity titration end point (M.S.50) in mice when these animals are infected with either influenza A or influenza B virus.

XII. Effects of Various Chemical Procedures on the Inhibitory Capacity of Fr.B Polysaccharide.—Polysaccharides can be subjected to a variety of procedures which will bring about alterations in their chemical structures. In the

present investigation the effects of oxidation by periodic acid and of hydroxyl ions on the activity of Fr.B polysaccharide with respect to mumps virus were studied.

Oxidation of the polysaccharide with periodic acid was carried out as follows: A solution of carbohydrate containing 0.4 gm. in 20 cc. was diluted with an equal volume of 0.5 M acetate buffer at pH 5.0. 30 cc. of 0.1 M HIO_4 was added, and the mixture diluted to 100 cc. At 10, 40, and 160 minutes, as well as at 24 hours, 25 cc. of solution was removed and 0.5 cc. of 50 per cent glycerol added to decompose the excess periodate. The solutions were thoroughly

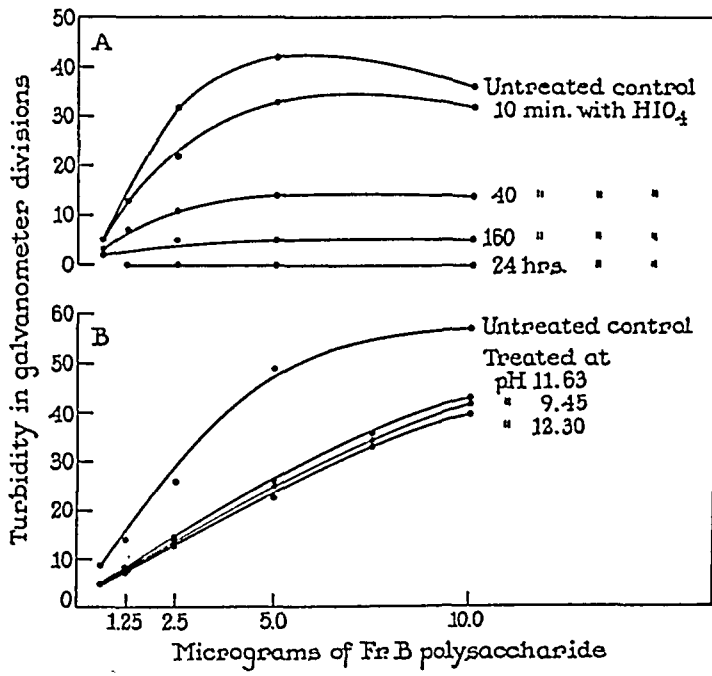

FIG. 4 *A*. Effect of treatment with 0.03 M HIO_4 at pH 5.0 on the specific serological activity of Fr.B polysaccharide. Solutions were mixed with immune rabbit serum diluted 1:20. Photometric observations were made 15 minutes after the mixtures were prepared.

FIG. 4 *B*. Effect of treatment with OH^- on the specific serological activity of Fr.B polysaccharide. Neutral solutions were mixed with immune rabbit serum diluted 1:15. Photometric readings were made 15 minutes later.

dialyzed against distilled water and the solid material recovered from the frozen state. In each instance a quantitative recovery was obtained.

Treatment of the polysaccharide with OH^- was performed by dissolving 20 mg. amounts of carbohydrate in 0.1 M borate buffers at pH 9.45, 11.63, and 12.30, respectively. After standing at 37°C. for 24 hours the borate was removed by dialysis against 0.9 per cent NaCl solution. Each solution was made to a final concentration of 5.0 mg. per cc. A further specimen was prepared by allowing a solution of carbohydrate to stand at pH 12.3 in an appropriate concentration of NaOH for 24 hours at 37°C. This solution was then neutralized with HCl but was not dialyzed.

Quantitative serological precipitation tests on solutions of the polysaccharide treated with periodate for various time intervals, and with OH^- at varying values of pH, were carried out photometrically as described above. The antiserum used was prepared by the immunization of rabbits with Friedländer bacillus type B.

The activity of these same solutions of polysaccharide with respect to mumps virus was determined in the following manner: Approximately 100 E.I.D. of virus was inoculated into the allantoic sac of each of a number of embryos and 3 hours later either unaltered Fr. B or a preparation of polysaccharide which had been subjected to chemical treatment was injected by the same route. Following the usual incubation period of 6 days the hemagglutination titer of each allantoic fluid was determined.

The results of quantitative precipitation tests with immune rabbit serum which are presented graphically in Fig. 4 *A* demonstrate that oxidation of Fr.B polysaccharide by 0.03 M HIO_4 at pH 5.0 brings about a rapid and progressive reduction in the specific serological activity of the carbohydrate. Indeed, at the end of a 24 hour period of contact with the reagent not only was there no serological activity demonstrable by the precipitation technique, but also the product of oxidation failed completely to inhibit specific precipitation by unaltered polysaccharide with homologous antiserum. This loss in serological activity, however, was not accompanied by any demonstrable reduction in biological activity with respect to mumps virus, as is shown below. In contrast to these results the effect of hydroxyl ions did not bring about destruction of serological activity (*cf.* Fig. 4 *B*), but completely abolished the biological activity of the carbohydrate as will be seen from the evidence presented below.

In Table XI are shown the results of experiments with chemically treated polysaccharide preparations and mumps virus in the chick embryo. It is evident that even after treatment with periodic acid for 24 hours the product of oxidation of the Fr.B polysaccharide was still capable of inhibiting multiplication of virus. It is also seen that, whereas treatment with OH^- at pH 9.45 did not affect the inhibitory activity of the polysaccharide, similar treatment at pH 11.63 completely inactivated the inhibitory effect of the carbohydrate. Polysaccharide treated with NaOH at pH 12.30 for 24 hours, but not dialyzed thereafter, was also found to have lost all capacity to inhibit multiplication of mumps virus. As is also shown in Table XI the aldobionic acid derived from Fr.B polysaccharide by acid hydrolysis had no effect upon multiplication of virus in the embryo even when as much as 5.0 mg. was injected.

The Fr.B polysaccharide preparation which was used throughout these experiments, although highly purified by repeated alcoholic precipitations, still contained 0.7 per cent nitrogen. It was of interest to determine what effect further purification would have upon the activity of the polysaccharide. The carbohydrate was therefore precipitated as its copper salt and after dissolving in a high concentration of acetate buffer was repeatedly precipitated with alcohol. The solution was then shaken with octyl alcohol-chloroform and finally electrodialyzed. The carbohydrate was recovered by freezing and drying and

its nitrogen content determined. The final product contained only 0.25 per cent nitrogen. When this more thoroughly purified preparation was tested in the chick embryo with mumps virus in the usual manner, it was found that its effectiveness in causing inhibition of virus multiplication was not changed. The fact that further purification did not diminish activity is important evidence indicating that the active substance is the polysaccharide itself and not an impurity associated with it.

TABLE XI

The Effect of Chemical Alterations of the Capsular Polysaccharide of Friedländer Bacillus Type B on Its Capacity to Cause Inhibition of Multiplication of Mumps Virus

1st injection Intra-allantoic	Interval	2nd injection Intra-allantoic	Specific serological activity	Amount of material injected	Hemagglutination titer of allantoic fluids* Log	Difference from control Log
E.I.D.	*hrs.*		*per cent‡*	*mg./embryo*		
MV 10^2	3	Fr.B	100	0.05	−0.48	−1.58
" "	"	Fr.B treated with HIO_4 160 min.	10	1.0	−0.59	−1.58
" "	"	" "		0.05	−1.43	−0.82
" "	"	Fr.B treated with HIO_4 24 hrs.	0	1.0	−0.39	−1.78
" "	"	" "		0.05	−1.28	−0.97
" "	"	Fr.B treated with OH^- pH 9.45 24 hrs.	50	0.1	−0.30	−2.09
" "	"	Fr.B treated with OH^- pH 11.63 24 hrs.	50	0.5	−2.25	−0.14
" "	"	" "		0.1	−2.53	+0.14
" "	"	Aldobionic acid (from Fr.B)	0	5.0	−2.41	+0.16

* Geometric mean of the hemagglutination titers.

‡ Approximate values (*cf.* Fig. 4).

DISCUSSION

The evidence obtained in this study indicates that capsular polysaccharides derived from type-specific Friedländer bacilli possess the capacity to cause inhibition of the multiplication of mumps virus in the allantoic sac of the chick embryo. These same polysaccharides are also capable of inhibiting the multiplication of pneumonia virus of mice (PVM) in the mouse lung (1,[1]). However, at least one of these carbohydrates, the capsular polysaccharide of Friedländer bacillus type B, shows no such inhibitory effect on the multiplication of Newcastle disease virus in the chick embryo or on the multiplication of influenza A and influenza B viruses either in the chick embryo or in the mouse lung.

Despite the fact that each of the five different viruses employed possesses the capacity to cause hemagglutination with appropriate erythrocytes, each

possesses in addition to immunological specificity other properties which serve sharply to differentiate one agent from another. It appears pertinent to point out that the viruses enumerated above can be separated into two groups on the basis of their rates of multiplication in susceptible hosts. Mumps virus and PVM (30) multiply at relatively slow rates in the chick embryo and the mouse, respectively, and both reach maximal titers in approximately 6 days. Influenza A (31), influenza B (32), and Newcastle disease (33) viruses multiply at relatively rapid rates in the chick embryo, as too do influenza A (34) and B viruses in the mouse, and each reaches maximal titers in approximately 2 days. It is of suggestive interest that among the viruses studied there appears to be a close correlation between the rate of multiplication and the capacity of the capsular polysaccharide of Friedländer bacillus type B to inhibit multiplication: viruses which multiply slowly are inhibited; those which multiply rapidly are not. It might be thought that the rapidity with which viruses in the latter group multiply is an adequate explanation for the fact that their multiplication is not inhibited by Friedländer polysaccharide. There is reason to think, however, that some other explanation is required. Even the injection of relatively large quantities of polysaccharide some hours before the inoculation of very small amounts of these viruses does not cause inhibition of their multiplication. These agents, in contrast to those which multiply slowly, appear to be capable of increasing in titer in a susceptible host regardless of the presence of a considerable concentration of Friedländer polysaccharide. It does not follow, however, that the viruses which multiply rapidly will necessarily continue to do so in the presence of other polysaccharides. Indeed, there is evidence indicating that with one of them (influenza A virus) apple pectin is effective as an inhibitor of multiplication when 25 to 50 mg. is injected into the allantoic sac (2). It will be recalled that this latter polysaccharide, in the quantity employed (2 mg.), showed no inhibitory activity even against very small amounts of mumps virus, which multiplies slowly.

In the light of all of these findings it seems reasonable to consider the possibility that both mumps virus and PVM require, in order that they may multiply, the presence of cellular metabolic systems which are not identical with those required for the multiplication of influenza A, influenza B, and Newcastle disease viruses. Were but a single chain of metabolic steps necessary for the multiplication of all of these agents, it would be expected that a polysaccharide which is capable of blocking multiplication of one should also prevent multiplication of the others. It appears evident that this is not the case.

The available evidence indicates that Friedländer polysaccharide does not induce its inhibitory effects as a result of direct action on mumps virus *per se*. Additional experimental evidence in support of this conclusion is presented in the accompanying paper (10). Previously it was shown that inhibition of multiplication of PVM (1) could not reasonably be explained by postulating a

direct effect of the same polysaccharide on the virus itself. It appears that with both agents the mechanisms of inhibition may be similar and dependent entirely upon metabolic alterations induced in the susceptible cells of appropriate hosts As earlier with PVM (1), so too with mumps virus (10) evidence has been obtained which indicates that the so called virus receptor of tissues susceptible to infection is not blocked by Friedländer polysaccharide. As a consequence, the first step in the initiation of infection, that is, the establishment of intimate contact between virus particles and susceptible cells, is undoubtedly accomplished even when much polysaccharide is present. It is, apparently, some later step in the process resulting in an increase in the number of virus particles which fails to function normally if Friedländer polysaccharide is present. That this step is one which is associated with the metabolic activities of the host cell, not the virus particle, seems very probable. Because the same polysaccharides which inhibit the multiplication of mumps virus in the allantoic sac also inhibit the multiplication of PVM in the mouse lung, it appears possible that but a single mechanism is involved. It is suggested that these polysaccharides block a metabolic step in the cells of both hosts, which step is required for the multiplication of both viruses.

The results obtained following chemical treatment of polysaccharide by the procedures described provide evidence indicating that the structural configuration which endows the Fr.B polysaccharide with specific serological activity is distinct from that which brings about inhibition of the multiplication of mumps virus and PVM (1). It should be pointed out that oxidation of the carbohydrate by periodic acid alters the molecule in such a way that the linkage between the carbon atoms of the individual hexose and hexoseuronic acid units bearing adjacent hydroxyl groups is severed without destroying the glycosidic unions. The hydroxyl groups themselves are oxidized to aldehydic groups. The resulting derivative may therefore be considered as a polyaldehyde (35). That the derivative has suffered no gross degradation is evident from the fact that it does not diffuse through cellophane membranes. It is difficult to explain why drastic chemical change of the polysaccharide molecule, such as that brought about by oxidation with periodic acid, fails to impair biological activity, whereas treatment with hydroxyl ions causes complete loss of the latter function. Although the two chemical reactions are by no means comparable, the fact remains that profound alteration in chemical structure is not necessarily accompanied by a change in biological activity. That alkali impairs but slightly the serological activity of the polysaccharide, but destroys completely its biological activity with respect to mumps virus, may be due to either depolymerization of the carbohydrate, enolization of terminal aldehydic groups, or to chemical changes of those groups which have a specific affinity for the susceptible cells of the host.

It is obvious that the hypothesis presented regarding the mechanism of action of polysaccharide inhibitors with respect to these two viruses is superficially similar to present ideas on the mechanism of action of certain chemotherapeutic agents on various microbial species. There is, however, a very fundamental difference. In the latter case the active substance appears to exert its effect by affecting the metabolism of the microbe *per se*. With viruses the point of attack is one step removed and it is probably the metabolic systems of the host cell, not the infectious agent, which are affected by polysaccharide.

SUMMARY

Polysaccharides derived from type-specific Friedländer bacilli cause inhibition of the multiplication of mumps virus in the allantoic sac of the chick embryo. As little as 5 μg. of polysaccharide is effective as an inhibitor. Inhibition of multiplication is obtained when polysaccharide is injected as long as 4 days after inoculation of virus. Chemical studies have shown that the structural configurations of the polysaccharide responsible for specific serological activity are not identical with those which determine the inhibitory effect relative to mumps virus. The possible mechanisms of the inhibition of viral multiplication by means of polysaccharides are discussed.

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THE EFFECT OF POLYSACCHARIDES ON THE REACTION BETWEEN ERYTHROCYTES AND VIRUSES, WITH PARTICULAR REFERENCE TO MUMPS VIRUS

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In the accompanying paper (1) it is shown that the capsular polysaccharides of type-specific Friedländer bacilli inhibit the multiplication of mumps virus in the allantoic sac of the chick embryo, yet have no demonstrable effect upon the multiplication of influenza A, influenza B, or Newcastle disease viruses. In an earlier report (2) it was demonstrated that polysaccharides of diverse origin, including the capsular polysaccharide of Friedländer bacillus type B, cause inhibition of the multiplication of pneumonia virus of mice (PVM) in the mouse lung. Because the active polysaccharides failed to cause inhibition of hemagglutination by PVM or to reduce the capacity of the normal mouse lung to combine with the virus (2), it was concluded that inhibition of the multiplication of PVM cannot be explained on the basis of blockade of virus "receptors." Recently, Green and Woolley (3) have reported that apple pectin inhibits the multiplication of influenza A virus in the allantoic sac of the chick embryo, and have found that the polysaccharide also causes inhibitions of hemagglutination by the virus. These workers suggested that inhibition of multiplication and hemagglutination of the virus by a polysaccharide might be closely related phenomena.

The results of preliminary experiments (4) with mumps, influenza A, influenza B, and Newcastle disease viruses indicated that the capsular polysaccharide of Friedländer bacillus type B causes inhibition of hemagglutination with each of the agents studied. Although polysaccharide-treated erythrocytes do not adsorb mumps virus, they do adsorb influenza A or B virus in a manner similar to untreated erythrocytes. There are evident discrepancies in the effect which polysaccharides exert on hemagglutination on the one hand, and on virus multiplication on the other. It appeared important, therefore, to carry out more detailed experiments, and to determine whether the inhibition of hemagglutination and multiplication of viruses by means of polysaccharides can be attributed to a single mechanism.

It will be shown that with mumps virus, as with PVM (2), there is no correlation between the capacity of polysaccharides to inhibit hemagglutination and

multiplication of the agent respectively. It will also be demonstrated that polysaccharides which fail to inhibit the multiplication of influenza A, influenza B, and Newcastle disease viruses markedly inhibit hemagglutination by these agents. Moreover, it will be shown that substances which block the effect of polysaccharides on the hemagglutination reaction with mumps virus do not have a similar action upon inhibition of virus multiplication. In addition, evidence will be presented which indicates that the mumps virus "receptors" present in the allantoic membrane of the living embryo are not blocked by active polysaccharide.

Materials and Methods

Viruses.—In these studies the following viruses were employed: mumps; influenza A, PR8 strain; influenza B, Lee strain; Newcastle disease; and PVM. The methods of cultivation and storage of each of these agents were identical to those already described (1, 5). Except for PVM each of the viruses was maintained by passage in the allantoic sac of the chick embryo; PVM was maintained by serial passage in the lungs of albino Swiss mice.

Hemagglutination Titrations.—Chicken, human group O, and mouse erythrocytes were in all instances freshly obtained, and washed 3 times with saline prior to their use. Hemagglutination titrations were performed as described in the accompanying paper (1).

Polysaccharide Preparations.—In most of the experiments the capsular polysaccharides of Friedländer bacilli type A (Fr.A), type B (Fr.B), and type C (Fr.C) (6, 7), respectively, were employed, although other polysaccharides previously studied (2) were also used. The products derived from the oxidation of Fr.B by periodic acid and by treatment with alkali as described in the accompanying paper (1) were also utilized.

Antiserum.—Sera from rabbits hyperimmunized with Friedländer bacillus type B (1) were used in specific precipitation experiments.

EXPERIMENTAL

It appears that with each of the agents employed in this study hemagglutination results from an interaction between the virus particles *per se* and erythrocytes. In order to determine the effect of polysaccharides on the hemagglutination reaction with these viruses, it was important to investigate the two components essential to the reaction as independently as possible. Therefore, certain experiments were designed to test the effect of treatment of virus with polysaccharide, while others were devised to test the effect of polysaccharide on erythrocytes. These experiments are described below.

I. The Effect of Polysaccharides on Mumps Virus.—The capacity of mumps virus to cause agglutination of chicken erythrocytes *in vitro* after the virus had been treated with various polysaccharides was determined. In the following experiment a large quantity of Fr.B was added to undiluted virus.

To 5 cc. of allantoic fluid infected with mumps virus was added 10 mg. of Fr.B dissolved in 1.0 cc. of saline. As a control, 1.0 cc. of saline alone was added to 5 cc. of infected allantoic fluid. This quantity of allantoic fluid was used because it is equal to the amount usually found in the allantoic sac of the embryos employed. The hemagglutination titer of each

mixture was determined immediately after preparation, following which the mixtures were held at 35°C. Aliquots were removed after 24 and 48 hours and their hemagglutination titers determined in duplicate in the usual manner.

As is shown in Table I it was found that there was no diminution in the capacity of mumps virus to cause hemagglutination even after holding the virus at 35°C. for 48 hours in the presence of 10 mg. of Fr.B. It should be pointed out that the quantity of polysaccharide employed in this experiment was 5 times greater than the largest amount used in experiments on the inhibition of multiplication of mumps virus *in vivo* and 2,000 times greater than the smallest quan-

TABLE I

The Hemagglutination Capacity of Mumps Virus after Treatment of a Constant Quantity with the Capsular Polysaccharide of Friedländer Bacillus Type B

Treatment of mumps virus				Results of hemagglutination test								Hemagglutination titer*
				Dilution* of allantoic fluid								
Mixture		Time	Temperature	4	8	16	32	64	128	256	512	
5.0 cc.	*1.0 cc.*	*hrs.*	°C.									
MV allantoic fluid	Saline	0		4	3	3	3	3	2	±	0	128
" " "	"	24	35	3	3	3	3	3	2	1	0	"
" " "	"	48	"	3	3	3	3	2	2	±	0	"
MV allantoic fluid	Fr.B 10 mg.	0		4	3	3	3	3	2	±	0	128
" " "	" "	24	35	4	3	3	3	3	2	±	0	"
" " "	" "	48	"	4	3	3	3	3	2	±	0	"

* Expressed as the reciprocal.

tity of Fr.B which is effective in causing inhibition of multiplication of the virus in the allantoic sac (1).

In order to determine whether a great excess of polysaccharide would affect the hemagglutinating capacity of mumps virus, additional experiments were performed in which decreasing amounts of virus were treated with large quantities of various polysaccharides.

Serial twofold dilutions of allantoic fluid infected with mumps virus were prepared in saline solutions of Fr.B in a concentration of 2.0, 5.0, and 10 mg. per cc., respectively. In addition, similar dilutions of virus were prepared in solutions containing 5 mg. per cc. of Fr.B which had been treated with OH^- at pH 12.3 for 24 hours at 37°C. (1) and in solutions of the capsular polysaccharide of pneumococcus type III. The mixtures were held either at room temperature or at 37°C. Immediately after preparation, as well as at 1, 2, and 3 hours, aliquots of each dilution were tested for their capacity to cause agglutination of chicken erythrocytes. Similar experiments were also carried out with influenza A, influenza B, and Newcastle disease viruses diluted in a saline solution containing 5 mg. per cc. of Fr.B.

The results of these experiments are shown in Table II. In no instance was the virus hemagglutination titer lower than that of the saline control when the erythrocyte suspension was added immediately after dilution of virus in the polysaccharide solution. When each of the diluted virus-polysaccharide mixtures was held at room temperature, no reduction in hemagglutination titer occurred during 2 hours, and only in the case of mumps virus did two- to fourfold reductions in titer occur after 3 hours. When mixtures were held at 37°C.

TABLE II

The Hemagglutinating Capacity of Various Viruses after Treatment of Decreasing Quantities with Different Polysaccharides

Virus	Treatment			Hemagglutination titer*	Hemagglutinating units of virus inhibited	Degree of inhibition of virus multiplication obtained *in vivo*‡ Log
	Dilutions of virus prepared in	Time	Temperature			
	5 mg./cc.	*hrs.*	°C.			
MV	NaCl (control)	3	37	128	—	
"	Fr.B	0		"	0	−1.98
"	"	1	23–25	"	"	
"	"	3	"	32	2	
"	"	1	37	"	"	
"	"	3	"	16	4	
"	Fr.B treated with OH^- at pH 12.3	"	"	"	"	−0.13
"	Pneumococcus type III	"	"	8	8	−0.73
IAV (PR8)	NaCl	"	"	512	—	
" "	Fr.B	"	"	"	0	−0.52
IBV (Lee)	NaCl	"	"	1024	—	
" "	Fr.B	"	"	"	0	−0.15
NDV	NaCl	"	"	"	—	
"	Fr.B	"	"	512	1	−0.07

* Expressed as the reciprocal.

‡ *Cf.* accompanying paper (1).

no significant decrease in titer was observed with any virus except mumps. After 1 hour at 37°C. in the presence of Fr.B mumps virus showed a reduction in hemagglutination titer of two- to fourfold, while after 3 hours the titer was reduced four- to sixteenfold. It will be noted that, when tested against decreasing amounts of mumps virus, Fr.B treated with OH^- showed as striking an effect as untreated Fr.B in causing a reduction of the hemagglutination titer of the virus despite the fact that this degradation product is completely incapable of causing inhibition of mumps virus multiplication (1). Likewise, the capsular polysaccharide of pneumococcus type III caused marked inhibition of hemagglutination by mumps virus under the conditions of these experiments,

but failed to cause significant inhibition of the multiplication of the virus in the allantoic sac (1). The product of oxidation of Fr.B by HIO_4 and the untreated capsular polysaccharides of Friedländer bacilli types A and C were not used because each substance itself caused agglutination of chicken red blood cells under the conditions of these experiments.

II. The Effect of Treatment of Erythrocytes with Polysaccharide.—The capacity of mumps virus to cause agglutination of chicken erythrocytes *in vitro* after the red blood cells had been treated with polysaccharide was determined. In a similar manner, the effect of such treatment of erythrocytes derived from various species upon hemagglutination with influenza A, influenza B, and Newcastle disease viruses as well as PVM was also studied.

Fresh suspensions of chicken, human group O, or mouse erythrocytes were prepared in saline as well as in saline solutions of various polysaccharide preparations. The concentration of erythrocytes employed was either 3 or 10 per cent. The various polysaccharides employed were: (1) Fr.B; (2) the product of oxidation of Fr.B by HIO_4 for 160 minutes (1); (3) degradation product of Fr.B following treatment with OH^- at pH 11.6 for 24 hours at 37°C. (1); (4) capsular polysaccharides of Friedländer bacilli types A (Fr.A) and C (Fr.C), respectively. The concentrations used were either 2 or 5 mg. per cc. The various erythrocyte suspensions were held at room temperature for at least 3 hours. In certain instances they were held at 4°C. for an additional 12 to 18 hours. Following this treatment, the erythrocytes were washed 2 or 3 times with saline. It was found, however, that washing was not an important step because the results were the same whether the erythrocytes were washed or not. Following treatment, the erythrocytes were diluted with sufficient saline to give 0.5 per cent suspensions. Hemagglutination titrations were then carried out with serial twofold dilutions of the following viruses; mumps, influenza A, influenza B, Newcastle disease, and PVM.

The results of experiments with erythrocytes treated with 5 mg. per cc. of Fr.B are shown in Table III. It was found that treated chicken erythrocytes were, in most instances, entirely inagglutinable by either mumps or influenza B virus. Similarly, chicken erythrocytes treated with Fr.B were agglutinated by both influenza A and Newcastle disease viruses at significantly lower virus dilutions than were control cells. It will be noted that mouse red blood cells similarly treated were agglutinated by as high a dilution of PVM as control erythrocytes. However, the treated cells were not agglutinated by as high a dilution of influenza A virus as were control cells. Influenza B virus consistently failed to agglutinate untreated mouse red blood cells. Human group O erythrocytes treated with Fr.B showed no diminution in agglutinability with either influenza A or influenza B virus. That the polysaccharide had been adsorbed by both human and chicken erythrocytes was readily demonstrated by agglutination tests with Fr.B immune rabbit serum. The treated erythrocytes, even though washed repeatedly, were markedly agglutinated by anti-Fr.B serum.

The results of an experiment with mumps virus and chicken erythrocytes treated with Fr.B, the alkali degradation product of Fr.B, the product of oxida-

tion of Fr.B with HIO_4, or the capsular polysaccharides of Friedländer bacilli types A and C, respectively, are presented in Table IV. Each of the polysaccharide preparations was used in a concentration of 2 mg. per cc. in the treatment of the red blood cells.

It will be noted that both unaltered Fr.B and the product derived from it after alkaline hydrolysis altered red blood cells sufficiently so that the hemagglutination titers obtained were significantly lower than the control. The product of oxidation with HIO_4 caused only a twofold reduction in titer, whereas

TABLE III

The Effect of Treatment of Erythrocytes with the Capsular Polysaccharide of Friedländer Bacillus Type B on Hemagglutination Titration End Points with Various Viruses

Virus	Erythrocytes*	Hemagglutination titer determined with		Hemagglutinating units of virus inhibited	Degree of inhibition of virus multiplication *in vitro*§ Log
		Control RBC	Fr.B-treated RBC‡		
MV	Chicken	128	0	128	−2.11
IAV (PR8)	Chicken	1024	64	8	
	Human	256	256	0	−0.52
	Mouse	1024	128	4	
IBV (Lee)	Chicken	1024	0	1024	
	Human	128	128	0	−0.15
	Mouse	0			
NDV	Chicken	4096	64	32	−0.07
PVM (heat-released)	Mouse	128	256	0	−1.97

* 0.5 per cent suspension.

‡ Erythrocytes were treated with 5 mg. per cc. of Fr.B for 3 hours at room temperature.

§ *Cf.* references 1 and 2.

the polysaccharides Fr.A and Fr.C caused no demonstrable effect. As is shown in the accompanying paper (1), Fr.A, Fr.C, and the product of oxidation of Fr.B with HIO_4 inhibit the multiplication of mumps virus in the allantoic sac as effectively as does untreated Fr.B; however, the product of alkaline hydrolysis of Fr.B does not.

The results of the experiments described above indicate that the treatment of erythrocytes with polysaccharide preparations may lead to a variety of effects as regards the agglutination of such red blood cells by viruses. The species from which the erythrocytes were derived, the particular polysaccharide

employed, as well as the nature of the chemical treatment to which it had been subjected, and the identity of the virus itself are all factors which require independent consideration. A change in any one of these variables, with no change in either of the others, so altered the results as to make systematic prediction virtually impossible.

III. Adsorption of Viruses by Polysaccharide-Treated Erythrocytes.—In view of the fact that certain polysaccharides reduced or eliminated the agglutinability of chicken erythrocytes by various viruses, it was of interest to determine whether this alteration was accompanied by a corresponding reduction in the ability of treated red blood cells to adsorb viruses. The results of preliminary

TABLE IV

The Effect of Treatment of Chicken Erythrocytes with the Capsular Polysaccharides of Type-Specific Friedländer Bacilli and Certain Degradation Products on Hemagglutination Titration End Point with Mumps Virus

Erythrocytes treated* with	Hemagglutination titer	Hemagglutinating units of virus inhibited	Degree of inhibition of virus multiplication obtained *in vivo*‡ Log
2 mg./cc.			
Nacl (control)	128		
Fr.B	16	4	−2.11
" treated with OH^- at pH 11.6	32	2	−0.14
" " " HIO_4	64	1	−1.58
Fr.A	128	0	−1.36
Fr.C	"	"	−2.06

* 3 per cent suspensions were treated for 3 hours at room temperature and then washed 3 times with saline.

‡ *Cf.* accompanying paper (1).

experiments (4) indicated that with influenza A and B viruses the extent of the reduction in these two properties was not proportional. With mumps virus, on the other hand, the capacity of treated erythrocytes to adsorb and to be agglutinated by the agent was correspondingly diminished.

Ten per cent suspensions of chicken erythrocytes were prepared in saline solution containing 5 mg. per cc. of Fr.B; control suspensions were prepared in saline containing no polysaccharide. The suspensions were held at room temperature for 3 hours and then stored at 4°C. for 12 to 18 hours. Shortly before use the erythrocytes were washed 2 to 3 times with saline. The packed red blood cells were then made to 10 per cent suspensions in undiluted allantoic fluid infected with either influenza A, influenza B, or mumps virus. The cell-virus mixtures were again held at room temperature and frequently shaken. At intervals aliquots were removed, centrifuged at 4°C., and the supernates withdrawn. The hemagglutination titers of all supernates obtained from a single experiment were determined simultaneously.

The results of typical experiments are presented graphically in Fig. 1. It was found that both influenza A and B viruses were adsorbed by and eluted from treated erythrocytes in a manner closely similar to their adsorption by and elution from control red blood cells. This result was particularly surprising in view of the finding that erythrocytes similarly treated showed greatly diminished agglutinability with these two viruses. In the case of mumps virus

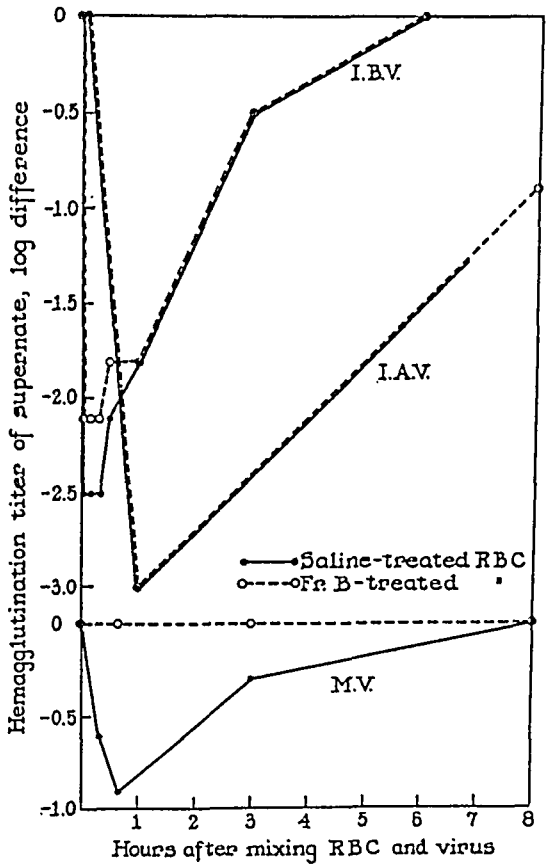

FIG. 1. The adsorption of influenza A (IAV), influenza B (IBV), and mumps (MV) viruses by chicken erythrocytes treated with the capsular polysaccharide of Friedländer bacillus type B.

it was found that Fr.B-treated erythrocytes failed entirely to adsorb demonstrable amounts of the agent, whereas control erythrocytes adsorbed most of the virus from the allantoic fluid.

Similar experiments were carried out with chicken red blood cells treated with the capsular polysaccharide of Friedländer bacillus type C (Fr.C). Such erythrocytes were capable of adsorbing mumps virus in a manner similar to control cells. It will be recalled that the agglutinability of red blood cells

treated with Fr.C also was undiminished when tested with mumps virus (*cf.* Table IV). It should be emphasized, however, that Fr.C was shown to be as effective an inhibitor of mumps virus multiplication as Fr.B (1).

The results of these experiments indicate clearly that polysaccharide-treated erythrocytes, even though completely inagglutinable by a particular virus, may or may not adsorb that virus. It appears of considerable interest that treated erythrocytes which failed to agglutinate visibly in the presence of more than 500 agglutinating units of influenza B virus were nonetheless capable of adsorbing the virus in apparently undiminished degree, whereas such red blood cells failed to adsorb mumps virus.

IV. Adsorption of Mumps Virus by the Allantoic Membrane.—The finding that chicken erythrocytes treated with Fr.B did not adsorb mumps virus *in vitro* raises the possibility that a similar effect might be demonstrable with the living chick embryo. Experiments were therefore carried out to determine whether mumps virus was adsorbed from the allantoic fluid by normal and polysaccharide-treated allantoic membranes.

Normal chick embryos 9 to 11 days of age were employed. About one-half the shell over the air sac was removed, and part of the shell membrane was torn away. All allantoic fluid possible was removed with a syringe and needle through an avascular area in the chorioallantoic membrane. Four cc. of saline solution containing 10 mg. per cc. of Fr.B was then injected into the allantoic sac. Control embryos likewise received 4 cc. of saline. After injection embryos were held at 35°C. for 3 hours. In some instances this period was prolonged to 12 to 14 hours. All fluid possible was again removed from the allantoic sac. Mumps virus was diluted either in buffered saline or in a saline solution of Fr.B, 10 mg. per cc., and 2.0 cc. was injected into the allantoic sac. The virus was diluted sufficiently to give the final fluid in the sac a hemagglutination titer ranging from 1:16 to 1:64. After thorough mixing of the fluid in the allantoic sac by drawing it back and forth into the syringe, 1.0 cc. was promptly removed. At intervals thereafter similar amounts were withdrawn. It was possible to obtain only a limited number of specimens, usually 3, from each embryo. The hemagglutination titer of each specimen was determined in the usual manner. In every instance it was possible to maintain the embryo in a living state throughout the entire experiment.

The results obtained in four separate experiments are presented graphically in Fig. 2. It was found that even when the allantoic sac had been treated for a number of hours with large quantities of Fr.B, mumps virus was nevertheless adsorbed from the fluid. Even when the allantoic membrane of the living embryo was treated with 40 mg. of Fr.B for 14 hours at 35°C. before injection of virus, and the latter diluted in a 1.0 per cent solution of the polysaccharide, no decrease in adsorption was demonstrable. It is obvious that these results are strikingly different from those obtained with chicken erythrocytes which had been treated with Fr.B *in vitro*. It appears that the polysaccharide, even in large amount, does not alter the living cells of the allantoic membrane as it does red blood cells, and that mumps virus "receptors" of allantoic membrane cells are not blocked by the presence of Fr.B.

V. The Effect of Ribonucleic Acid on the Activity of Polysaccharide (Fr.B).— In an attempt to find a substance which would neutralize or block the inhibitory action of polysaccharide (Fr.B) with respect to the hemagglutinating activity of mumps virus, the effect of ribonucleic acid was studied.

A solution of commercial yeast nucleic acid (Boehringer) having a concentration of 50 mg. per cc. in saline was prepared, and the pH adjusted to 7.1. The solution was sterilized by filtration. Equal volumes of nucleic acid solution and a solution of Fr.B, 10 mg. per cc. in saline, were mixed and held at room temperature for 15 minutes before use. Chicken erythrocytes were treated with the mixture as described above. The capacity of mumps virus to agglutinate treated red blood cells was then determined.

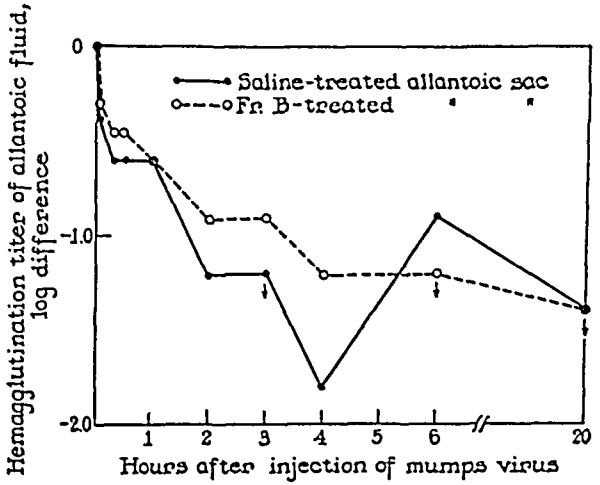

FIG. 2. The rate of adsorption of mumps virus by the living allantoic membrane following treatment with the capsular polysaccharide of Friedländer bacillus type B (Fr.B).

The results of a typical experiment are shown in Table V. It is seen that, although chicken erythrocytes treated with Fr.B alone were not agglutinated by mumps virus, cells treated with a mixture of the polysaccharide and ribonucleic acid were agglutinated by almost as small quantities of virus as control erythrocytes. Treatment with ribonucleic acid alone had no effect on the hemagglutination reaction.

Adsorption and elution of mumps virus with chicken erythrocytes previously treated with a mixture of Fr.B and ribonucleic acid were also studied. The results are presented graphically in Fig. 3. It was found that the virus was adsorbed and subsequently eluted from the treated red blood cells in a manner closely similar to that observed with control erythrocytes. Cells treated with Fr.B alone failed to adsorb mumps virus. In other experiments it was found that after red blood cells had been treated with Fr.B, the addition of ribonucleic acid in amounts as large as 50 mg. per cc. failed to cause dissociation of the

erythrocyte-polysaccharide combination. Cells treated in this manner remained inagglutinable by mumps virus.

Because ribonucleic acid blocked the *in vitro* activity of polysaccharide, it appeared of interest to determine whether this substance would also neutralize the *in.vivo* activity of Fr.B with respect to mumps virus. The effect of a mixture of Fr.B and nucleic acid on the multiplication of virus in the allantoic sac was therefore studied.

TABLE V

Hemagglutination Titrations with Mumps Virus and Chicken Erythrocytes Treated with the Capsular Polysaccharide of Friedländer Bacillus Type B and Ribonucleic Acid.

Erythrocytes treated* with		Hemagglutination titer
Fr.B	Ribonucleic acid	
mg./cc.	*mg./cc.*	
0	0	128
5	0	0
"‡	25‡	64
0	"	128

* Treated for 3 hours at room temperature and then washed 2 times with saline.

‡ These substances were mixed and held at room temperature for 20 minutes before erythrocytes were added.

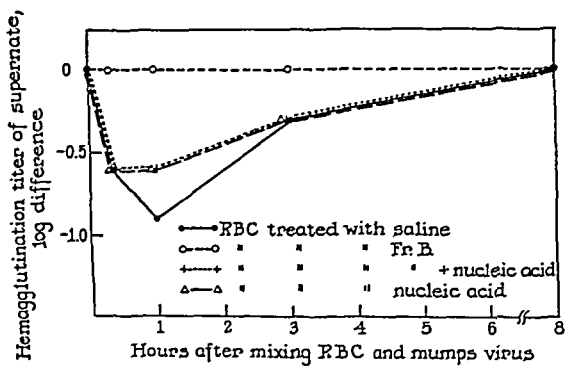

FIG. 3. The rate of adsorption of mumps virus by chicken erythrocytes following treatment with the capsular polysaccharide of Friedländer bacillus type B (Fr.B) and ribonucleic acid.

Saline solutions of Fr.B containing from 10 to 0.5 mg. per cc. were mixed with equal volumes of solutions of ribonucleic acid, 50 mg. per cc. After a period of 20 to 30 minutes at room temperature, 0.2 cc. of each mixture was injected into the allantoic sac of 7 to 9 day old embryos. Each embryo received from 0.05 to 1.0 mg. of Fr.B and 5 mg. of nucleic acid. In some experiments a mixture containing 2 mg. of Fr.B and 10 mg. of ribonucleic acid was injected. After an interval of 3 hours, either 10 or 100 E.I.D. of mumps virus was inoculated into the allantoic sac. The embryos were incubated at 35°C. for 6 days. They were then chilled overnight

at 4°C., the allantoic fluid removed, and the hemagglutination titer of each fluid determined (1). At least 4 embryos were used in each group, and at least 2 groups of control embryos were employed in each experiment.

The results are shown in Table VI. It will be noted that ribonucleic acid had no effect on the capacity of the polysaccharide to inhibit the multiplication of mumps virus even when 100 times more nucleic acid than Fr.B was employed.

The results of these experiments indicate clearly that ribonucleic acid is capable of blocking the inhibitory effect of polysaccharide (Fr.B) on the hemagglutination reaction with mumps virus, but has no blocking action on the inhibitory effect of Fr.B on multiplication of the virus.

TABLE VI

The Effect of the Capsular Polysaccharide of Friedländer Bacillus Type B and Ribonucleic Acid on the Multiplication of Mumps Virus in the Chick Embryo

1st injection Intra-allantoic	Interval	2nd injection Intra-allantoic	Incubation at 35° C.	Hemagglutination titer of allantoic fluids Log	Difference from controls Log
mg./embryo	*hrs.*	*E.I.D.*	*days*		
Fr.B, 2 mg.	3	MV 10	6	0	−1.52
Fr.B, 0.05 mg.	"	" 10^2	"	−0.99	−1.41
Fr.B, 2 mg. + ribonucleic acid, 10 mg.	"	" 10	"	0	−1.52
Fr.B, 0.05 mg. + ribonucleic acid, 5 mg.	"	" 10^2	"	−1.03	−1.37
Ribonucleic acid, 10 mg.	"	" 10	"	−1.81	+0.29
" " 5 "	"	" 10^2	"	−2.16	−0.24

DISCUSSION

The results of the experiments presented in this communication and those described in the accompanying paper (1) indicate clearly that there is no correlation between the effects of polysaccharides on the hemagglutination reaction with viruses and their inhibitory action on the multiplication of viruses. Had a definite correlation been demonstrated, it would have been possible to put forward a relatively simple hypothesis regarding the probable mechanism of the inhibitory action of polysaccharides with respect to virus multiplication.

Not only do some polysaccharides which markedly inhibit hemagglutination by one virus *in vitro* fail entirely to inhibit multiplication of the same virus *in vivo*, but others which are highly effective as inhibitors of the multiplication of of a virus are without discernible effect on the hemagglutination reaction with the same agent. Green and Woolley (3) also noted that some carbohydrates which inhibited hemagglutination with influenza A virus did not reduce multiplication of the virus.

The results obtained with the Friedländer type B polysaccharide after chemical alterations provide strong support for the concept that inhibition of hemagglutination and inhibition of multiplication of viruses are not closely related phenomena. Certain chemical changes induced in the carbohydrate, as for example treatment with OH^-, entirely abolish inhibitory activity with respect to virus multiplication without markedly diminishing the capacity of the carbohydrate to inhibit viral hemagglutination. Other chemical alterations, such as treatment with HIO_4, almost eliminate inhibitory activity with respect to hemagglutination but leave unaffected the capacity to inhibit multiplication of the agent. In the light of these observations it appears improbable that, with respect to inhibition by a polysaccharide, the erythrocyte component which reacts with a virus is closely related to the constituents of susceptible tissue cells essential for multiplication of that virus.

De Burgh and his coworkers (8) have recently shown that one component of human erythrocytes which reacts with influenza A virus is, in large part, polysaccharide. The nature of the component of susceptible tissue cells which appears to react with influenza A virus (9) in a similar manner is not yet known. However, evidence has been obtained indicating that the component present in normal mouse lung tissue which reacts with PVM is not polysaccharide but probably protein in nature (10). Nonetheless, the multiplication of PVM in the mouse lung is inhibited by certain polysaccharides (2).

Were the inhibition of hemagglutination to be satisfactory as a model for the study of the mechanism by which virus multiplication is inhibited *in vivo*, it appears necessary that inhibition of hemagglutination be associated with inhibition of adsorption of viruses by erythrocytes. It is evident from the results obtained that these two properties are not correlated, for some polysaccharides completely inhibit hemagglutination, yet have no demonstrable effect upon adsorption of the same virus by erythrocytes. Had these properties been associated, it would have been necessary to show that adsorption of virus by susceptible tissue cells is prevented by polysaccharide before the validity of the erythrocyte-virus model could be assumed. With mumps virus in the allantoic sac, as also with PVM in the mouse lung (2), it appears that such is not the case. In both instances, despite the presence of relatively large amounts of a polysaccharide highly active as an inhibitor of virus multiplication, apparently unaltered adsorption of virus by tissue cells occurs. Thus, the first step in the establishment of infection with a virus, *i.e.* combination or union between virus particles and susceptible cells, is not prevented by polysaccharides which are active as inhibitors of virus multiplication.

SUMMARY

Polysaccharides which cause inhibition of the multiplication of mumps virus in the allantoic sac may or may not cause inhibition of hemagglutination by

the virus. Moreover, such substances may or may not prevent adsorption of the virus by erythrocytes. The available evidence indicates that polysaccharides active as inhibitors do not block adsorption of mumps virus by cells of the living allantoic membrane. With influenza A, influenza B, and Newcastle disease viruses, as well as with PVM, there also appears to be a lack of correlation between the *in vitro* and *in vivo* inhibiting activity of polysaccharides.

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AN OSMOTIC SYSTEM WITHIN THE CYTOPLASM OF CELLS*

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PLATES 6 TO 8

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A study of the cytoplasm of cells of normal organs and of tumors has given evidence that it consists in great part of discrete particles, cytochondria, with surface properties that make them permeable to water and to other substances (1). They have an outer rim that takes up stains and a clearer or unstained center. Basophile substance which is demonstrably ribonucleic acid (2) in liver cells and some other tissues may accumulate at their surface, perhaps by adsorption. Of the bodies that I have designated cytochondria, only a part have the characteristic staining reactions of mitochondria. The latter in the presence of certain solvents or as the result of pathological changes such as those accompanying chloroform poisoning or with the transformation of liver cells into cells of hepatoma lose their distinctive reactions and become apparently indistinguishable from other cytochondria.

When liver or kidney tissues are immersed in distilled water or in hypotonic solutions, the cytochondria, including mitochondria, swell and become spherical bodies, now readily recognizable by high power magnification. With further swelling, the swollen bodies are crowded against one another so that their stained rims and clear centers give a foam-like appearance to the cytoplasm. The purpose of the present study is to define some of the conditions under which these bodies undergo osmotic swelling.

Methods

The occurrence of minute intracellular bodies with surface properties that bring about osmotic changes within the cytoplasm has suggested the need of a quantitative method by which the intake of water may be compared with coincident changes in these cytoplasmic structures. The method introduced by Phillips, Van Slyke, and their coworkers (3) for determination of the specific gravity of the plasma has been found to be adaptable to the measurement of changes in the specific gravity of parenchymal tissues under various conditions.

Small pieces of tissue have been inserted below the surface of copper sulfate solutions with graded specific gravity. The preparation of these solutions has been described in detail in the publication cited. I have found it convenient to use solutions of copper sulfate in quanti-

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ties of 100 cc. varying in specific gravity from 1.010 to 1.110 and differing in succession by 5 units. Standardized hydrometers have been used to test the specific gravity of the solutions that have been used and to determine when they have become altered by repeated use.

Slices of liver or other tissue have been cut with a razor blade about 0.5 mm. in thickness and 5 or 6 mm. across and immersed in different fluids. At intervals, shortest during the early period of immersion, a slice has been removed from the fluid and its specific gravity determined. Excess of fluid has been removed by applying the slice to the surface of cotton gauze, the flat surfaces of the slice being applied to the gauze at least twice. Small particles of the tissue, about 1 mm. across, cut with the razor blade from the slice, are used for the tests. They have been at first introduced below the surface of the copper sulfate solution by a finely pointed stainless steel forceps and gently forced from the forceps by a narrow platinum spatula but later a special device has been used to launch the particle below the surface of the copper sulfate solution with the least possible momentum. A straight platinum wire fixed in a holder passes through two loops, one at the end and the other about 1 cm. from the end of a second wire fixed in another holder. The tissue particle brought into contact with the end of the straight wire adheres to it and can be inserted below the surface of the copper sulfate solution. The lower loop of the other wire guided by the upper loop is used to push the particle of tissue from the end of the straight wire. It is essential that the particle be projected into the fluid with the least possible momentum because the particle rises or sinks with relation to its own specific gravity in the short interval before its density is changed by contact with the test fluid. If the particle rises only a millimeter or two at this time, its specific gravity is evidently less than that of the solution. The specific gravity of the particle has been recorded as that of the least concentrated solution of copper sulfate in which it rises.

For comparison with water intake of tissues after immersion in different fluids, as determined by their specific gravity, changes in the cytoplasm of cells have been studied in tissue fixed after removal at intervals from the immersion fluid. Tissues were from white rat or guinea pig and when not otherwise designated were from the rat. Fixation in a mixture of lanthanum acetate (tenth molar) and formalin (10 per cent) as described in an earlier publication (1) has been used because mitochondria are distinguishable from other cytochondria which do not give the mitochondrial stain, the former staining with the basic dye (methylene blue with azure II) and the latter with the acid dye (phloxin or rose bengal). The basophile substance which forms clumps in the liver cells and gives the reactions of ribonucleic acid is well defined by the Giemsa stain after fixation in lanthanum acetate and formalin or in Zenker's fluid with or without acetic acid. Mitochondria have been stained by aniline fuchsin or by iron hematoxylin after fixation in Regaud's fluid. It is noteworthy that methylene blue, after fixation in lanthanum acetate, and formalin and iron hematoxylin, after fixation in Regaud's fluid, stain both mitochondria and the basophile substance of the cytoplasm whereas aniline fuchsin by the usual procedure stains mitochondria alone.

The changes that are produced by the penetration of fluids into excised pieces of tissue, as might be expected, are not uniformly distributed. Immediate and often severe changes occur at the surface where the fluid, unchanged by passage through the tissue, comes into contact with surviving cells. The injury which ensues doubtless modifies the further penetration of the fluid. In the earliest period of immersion, cellular changes are almost wholly limited to a superficial zone but when ultimately the interior of the tissue is implicated, the subsequent changes tend to be uniform.

The changes that accompany immersion are doubtless in part supravital but the difficulty of determining when, during their progress, death of cells occurs is considerable. All of the fluids that have been used, including Ringer's solution and even blood serum, produce conspicuous changes in the tissue.

Changes in Liver Immersed in Water

When liver tissue in thin slices is immersed in distilled water, its specific gravity falls rapidly during the first 15 minutes, decreasing from the normal level of liver tissues, between 1.090 and 1.100, to approximately 1.055 (Text-figs. 1 to 3, 7 to 10). During the next 15 minutes, decrease of specific gravity is less rapid and during the next half hour still slower. After 2 hours, an approximate equilibrium is established and the specific gravity of the immersed tissue is maintained at 1.025 or somewhat less. A slight diminution of specific gravity may occur after 3 hours in water.

Liver immersed in water undergoes immediate change with considerable evidence of injury in a narrow zone about the surface of the slice of tissue. After from 10 to 15 minutes of immersion, the cytoplasm of cells about the edges of the tissue becomes foam-like and is no longer stained by the basic dye (Fig. 1, *A*). Mitochondria here are not stained and the clumped basophile substance of the cytoplasm (ribonucleic acid, 2) has disappeared. The cytoplasm is stained only by the acid dye (pink with rose bengal). The cells at the outermost margin of the tissue show more evidence of injury than those in the deeper part. Here the cytoplasm of cells does not have the uniform foam-like appearance seen elsewhere but the vacuolar spaces that give the foam-like appearance are irregular in size and shape and the partitions between them are fragmented in places. The nuclei of these cells stain faintly, the chromatin is in fine particles, and the nuclear membranes are thin and indented.

Next to this outer acidophile zone and in places with an abrupt margin is a zone (Fig. 1, *B*) in which material stained by the basic dye is apparently concentrated and deeply stained. Some cells are homogeneously stained but most of them have cytoplasm that is foam-like as in the outer zone but with deeply stained basophile substance in the interstices between the clear spaces (Fig. 7). With the Giemsa stain (which does not stain mitochondria) the basophile substance is well stained.

At the same time changes are in progress in a third zone (Fig. 1, *C*) which merges with the unchanged interior of the tissue. In this zone, cells are swollen and their outlines are sharply defined. With the mitochondrial stain (methylene blue after fixation in lanthanum acetate and formalin) mitochondria may be spherical and much swollen (Figs. 12 and 13). Some of the swollen mitochondria may have a vesicular appearance with a center lightly stained or unstained and a narrow rim of mitochondrial stain (not defined in the photographs; see diagram Fig. 14, *c*). It is evident that the mitochondria are losing their

distinctive reaction but they remain as spherical bodies stained by the acid dye. These have a vesicular appearance because their rims stain more deeply than their centers (see diagram Fig. 14, *d*). In many cells mitochondrial stain has disappeared almost completely but, presumably in a transitional stage, a minute round particle with mitochondrial stain may be seen at the periphery of the acidophile body (Fig. 14, *e*).

The changes that occur at the surface of the slice of liver extend more deeply during 1 hour of immersion (Fig. 2) but are otherwise similar to those that have been described. The changes of the innermost zone (Figs. 1 and 2, *C*) spread throughout the interior of the immersed tissue. No normal mitochondria are visible and the swollen cells contain swollen vesicular bodies. These bodies are at first discrete (Fig. 5) but later, after about 2 hours, pressed against one another, they give an almost uniform foam-like appearance to the cytoplasm (Fig. 6).

The basophile substance which forms a conspicuous peripheral zone (Figs. 1 and 2, *B*) in the early period of immersion is diminished after 1 hour and has disappeared after 2 or 3 hours. Basophile substance in the interior of the tissue remains for a time as stained material irregularly distributed in the interstices between the swollen cytochondria (Fig. 6) but after 4 hours has disappeared.

Swelling of cells and of cytochondria reaches its height after 2 hours and the foam-like appearance of cytoplasm that results has an almost uniform appearance but later, vacuoles are often large and irregular. Diminution in the width of liver cell columns with corresponding increase of the sinusoidal spaces between them suggests that rupture of distended cytochondria may have permitted some escape of accumulated fluid.

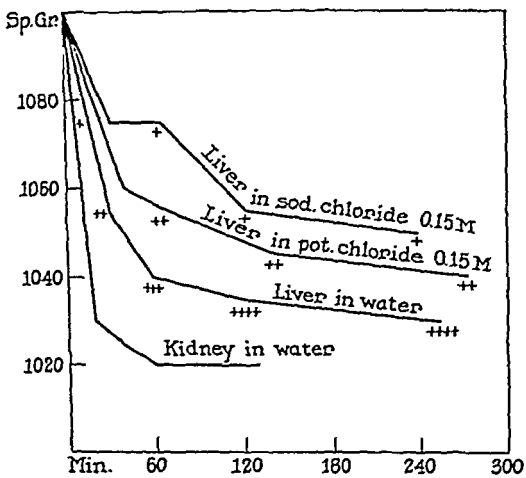
It has seemed convenient to indicate by a series of plus signs the succession of changes that occur in the particulate bodies of the cytoplasm as the result of increase of their water content. The immediate effect of water immersion is the appearance about the margin of the tissue of a narrow zone in which cytoplasm of cells has lost its basic stain and has assumed a foam-like appearance. In the adjacent cells cytochondria (including mitochondria) are moderately swollen but in the interior of the tissue, neither cells nor cytochondria are enlarged. This stage has been designated one plus (+). With widespread entrance of water into the tissue, cytochondria are moderately swollen, not only at the edges but also in the interior of the slice of tissue (two plus, ++). With further intake of water, cytochondria are seen as much enlarged spherical bodies usually within swollen cells (three plus, +++). When cytochondria are so swollen that they are in contact with one another the cytoplasm has a foam-like appearance (four plus, ++++). These signs on the graphs showing specific gravity indicate the associated changes in the cytoplasm.

Changes in Liver Immersed in Solutions of Sodium Chloride

When liver tissue is immersed in solutions of sodium chloride, change of specific gravity decreases with the concentration of the solution. In Text-

figs. 1 and 2 the specific gravity of liver immersed in 0.15 molar sodium chloride (slightly more than 0.85 per cent) is compared with that in distilled water. In the former, it diminishes slowly and reaches an approximate equilibrium at a level much above that after water immersion. When the concentration of sodium chloride is increased to 0.3 molar, there is scant intake of water and with greater concentration, water is lost.

When liver is immersed in 0.15 molar sodium chloride, the zone of foam-like cytoplasm so promptly formed at the surface of tissue in water is not seen, but within 15 minutes the basophile material of the cytoplasm has disappeared from a peripheral zone (Fig. 3, *A*). Here the mitochondria retain their usual



TEXT-FIG. 1. Changes in specific gravity: (*a*) of liver immersed in water; (*b*) in 0.15 molar solution of sodium chloride; (*c*) in 0.15 molar potassium chloride; (*d*) of kidney immersed in water. The abscissa gives the period of immersion in minutes. Plus signs, as explained in the text, indicate variations in the swelling of particulate bodies (cytochondria) of the cytoplasm. In the figures representing specific gravity the decimal point has been omitted.

size and shape but may take a somewhat modified stain (violet instead of blue with methylene blue after fixation in lanthanum acetate and formalin). Liver cells are diminished in size so that the columns of them are narrower than usual, with wide sinusoidal spaces between them. (These changes are seen in Fig. 9 still persisting after 3 hours of immersion.) At the outermost margin, the cells appear to have undergone severe injury, for their cytoplasm is fragmented, the chromatin of the nuclei is diminished, and nuclear membranes are thin and wrinkled. Below the peripheral zone in which liver cells have lost their basophile stain is an irregularly disposed zone in which cells are conspicuously basophile because the clumped basophile material appears to be overstained (Fig. 3, *B*). During the period from 1 to 3 hours of immersion, the two zones

may occupy a considerable part of the sectioned tissue. In the interior of the tissue, mitochondria persist longer than with water immersion. Some become acidophile and unchanged in size but after from 3 to 5 hours, they may be slightly swollen.

When liver is immersed in stronger solutions of sodium chloride, namely, 0.3 and 0.4 molar, the mitochondria in wide areas immediately below the surface retain their usual form and a modified stain even after 5 hours. Further from the surface, cells containing basophile material in conspicuous clumps are seen in sharp contrast with cells that have lightly stained mitochondria and no intervening basophile material. After 3 hours mitochondrial stain in the interior of the tissue is lost in spots, basophile material has disappeared, and there is slight swelling of cytochondria.

Changes in Liver Immersed in Solutions of Potassium Chloride

When liver is immersed in a 0.15 molar solution of potassium chloride, its specific gravity falls more rapidly than in a corresponding solution of sodium chloride, and approximate equilibrium is established at a lower level (Text-fig. 1). Changes in the tissue resemble in general those in solutions of sodium chloride of corresponding strength. Preservation of mitochondria as recognizable bodies, usually with somewhat modified stain, is widespread. The cells in which this change occurs are evidently shrunken and the cell columns they form are narrow. Nevertheless, in the interior of the tissue, after from 3 to 5 hours of immersion, spots occur in which cells are swollen, mitochondrial stain is lost, and the remaining acidophile bodies are swollen. This swelling seems to occur earlier and becomes greater than in the corresponding concentration of sodium chloride but the difference is not conspicuous.

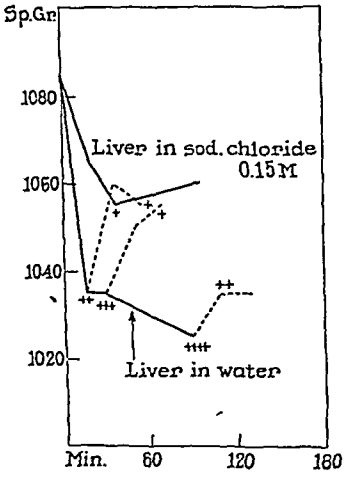
With stronger solutions of potassium chloride, 0.3 and 0.4 molar, the mitochondria remain in large part well defined during 3 hours but after 5 hours the tissue is almost wholly acidophile and there is considerable swelling of cytochondria.

Changes in Liver Immersed in Solutions of Calcium Chloride

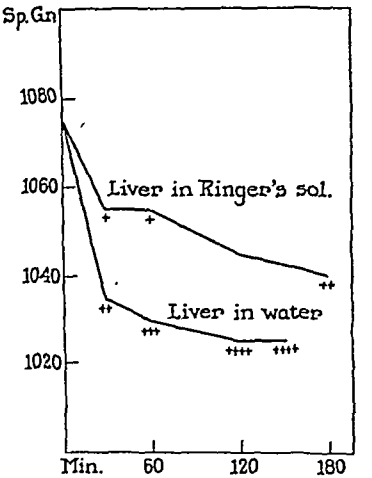
Following immersion in 0.15 molar calcium chloride, the changes in specific gravity resemble those in sodium chloride of the same molar concentration and equilibrium is reached at approximately the same level. Nevertheless, the changes in the tissue are not identical. There is a well defined zone at the edge of the tissue where mitochondria and basophile substance have disappeared and here, as after water immersion, cytochondria are so much swollen that the cytoplasm is foam-like. Below this acidophile zone, a sharply defined and deeply basophile zone is formed, but after 3 or 4 hours this has disappeared and the tissue is almost wholly acidophile. Throughout the tissue, though cells are not conspicuously enlarged, the cytochondria are much swollen.

Changes in Liver Immersed in Ringer's Solution

Liver tissue has been immersed in the modification of Ringer's solution known as Krebs-Ringer solution (4) and widely used in the manometric determination of supravital oxygen consumption of tissues. The electrolytes it contains are each adjusted to a molar concentration of 0.15, approximately isotonic for red blood corpuscles, and presumably they may be used in varied proportions with no significant deviation from isotonicity. A phosphate buffer with pH 7.4 is added to the solution. When slices of liver are immersed in this solution their specific gravity falls rapidly and reaches an equilibrium at



TEXT-FIG. 2.



TEXT-FIG. 3.

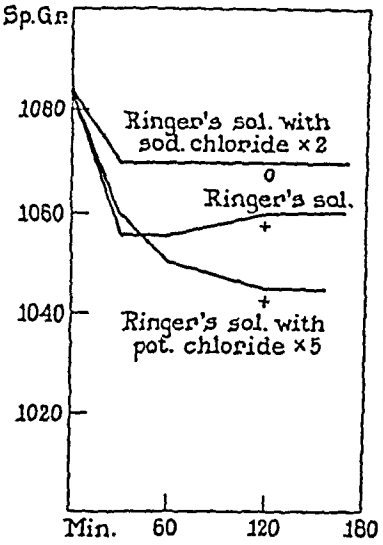
TEXT-FIG. 2. Specific gravity of liver of guinea pig immersed: (*a*) in water and (*b*) in 0.15 molar sodium chloride. The broken lines indicate the changes following transfer of liver tissue from water to the solution of sodium chloride.

TEXT-FIG. 3. Changes in the specific gravity of rat liver and swelling of cytochondria following immersion: (*a*) in water and (*b*) in Ringer's solution.

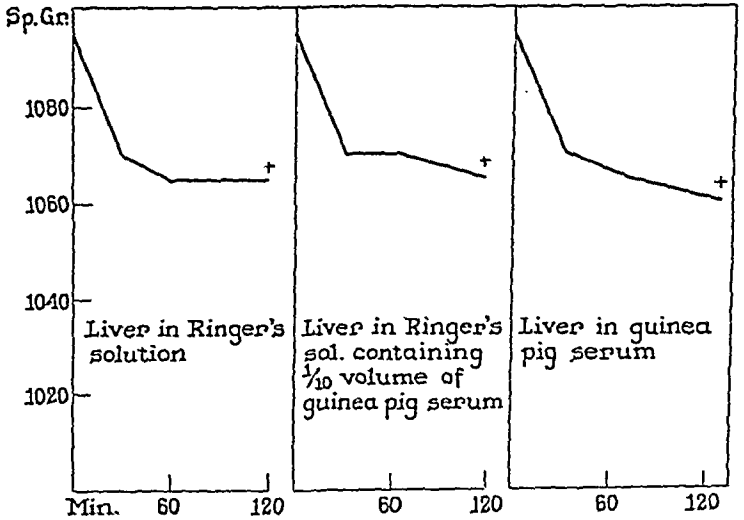
a level which is approximately the same as that in a 0.15 molar solution of sodium chloride (Text-figs. 3, 4, 7, 8, and 10).

Immediately after immersion of liver tissue in Ringer's solution, basophile substance and in large part the mitochondria disappear from cytoplasm of cells in a zone, sometimes of considerable breadth, about the edge of the tissue (Fig. 4, *A*). In a few places shrunken cells near the surface contain mitochondria with altered (violet) stain but cells in this outer zone are in general conspicuously acidophile with swollen cytochondria which give a foam-like appearance to the cytoplasm. A very sharp line of demarcation separates the acidophile zone from a deeply basophile zone below it (Fig. 4, *B*). Here basophile sub-

stance next to the outer zone gives an intense diffuse stain to the cytoplasm but further inward, clumped basophile substance and mitochondria are distinguish-



TEXT-FIG. 4. Specific gravity of liver: (*a*) in Krebs-Ringer solution, (*b*) in the same solu tion with twofold increase of sodium chloride, and (*c*) in the same solution with fivefold in crease of potassium chloride.

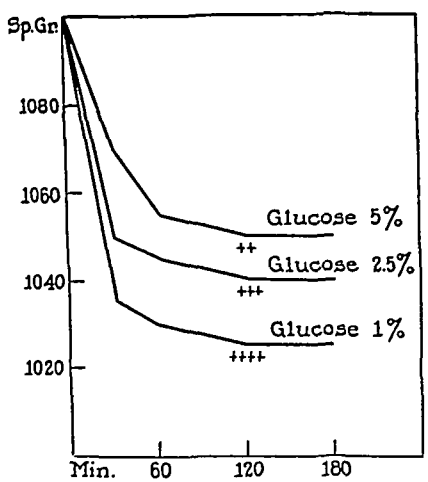


TEXT-FIG. 5. Specific gravity of liver of guinea pig: (*a*) in Krebs-Ringer solution; (*b*) in the same solution with one-tenth of its volume replaced by serum of guinea pig; (*c*) in blood serum of guinea pig.

able. The results have been the same when the phosphate buffer (pH 7.4) has been omitted from the modified Ringer's solution that has been used. The changes described are still present after 1 or 2 hours but at the end of this time,

in the interior of the tissue, mitochondria are not stained and the acidophile cytochondria have undergone some swelling (two plus in Text-figs. 3 and 7).

When the quantity of sodium chloride in Ringer's solution has been increased twofold, entrance of water into the tissue has been less and equilibrium has been reached at a higher level. When the potassium chloride has been increased five times, though the molar concentration of the solution remains the same, equilibrium, on the contrary, is at a lower level (Text-fig. 4). When guinea pig liver tissue has been immersed in a solution in which one-tenth of the volume of Ringer's solution has been replaced by serum of guinea pig or in the latter undiluted, the changes in specific gravity have been almost identical with those in Ringer's solution and, in both instances, there has been considerable intake of water (Text-fig. 5).



TEXT-FIG. 6. Specific gravity of rat liver and cytochondrial changes in 1, 2.5, and 5 per cent glucose.

Changes in Liver Immersed in Solutions of Glucose and Sucrose

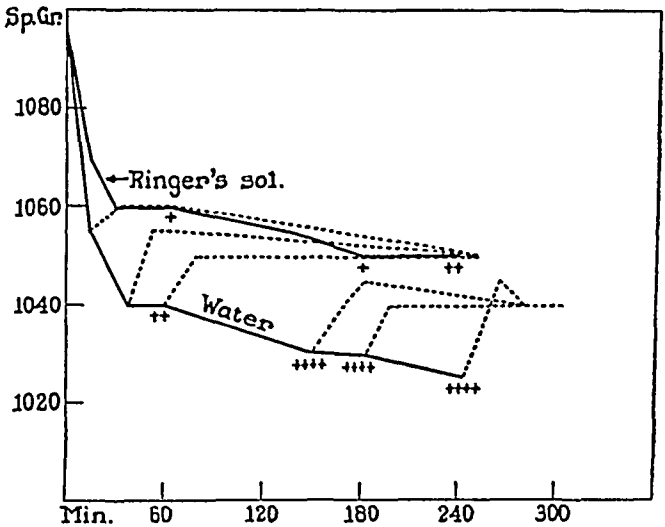
In 1 per cent glucose solution, the changes in specific gravity differ little from those in distilled water (Text-fig. 6) whereas in 5 per cent solution, they approximate those in Ringer's solution. After 1 hour in the former, the cytoplasm has the uniform foam-like appearance seen after water immersion, but in 5 per cent solution, cytochondria are only slightly swollen.

Changes of specific gravity in solutions of sucrose are similar to those in solutions of glucose. In 0.15 molar solution, intake of water is considerably less than in liver immersed in distilled water, but swelling of cytochondria is almost as great. In 0.2 and 0.3 molar solutions, intake of water and swelling of cytochondria are diminished. In a solution of molar strength, liver tissue

loses water and cytochondria are swollen only at the surface where the solution is in immediate contact with the tissue.

Changes Following the Transfer of Liver from Water to Hypertonic Solutions

When specific gravity has diminished following immersion in water, the change may be reversed by placing the tissue in a solution of sodium chloride or in Ringer's solution. If the transfer to 0.15 molar solution of sodium chloride is made after water immersion during 15 minutes, specific gravity returns to a level about the same as that of tissue kept for a corresponding time continuously in this solution (Text-fig. 2). The resulting increase of specific gravity occurs promptly and with few exceptions reaches a maximum after about 15 minutes.

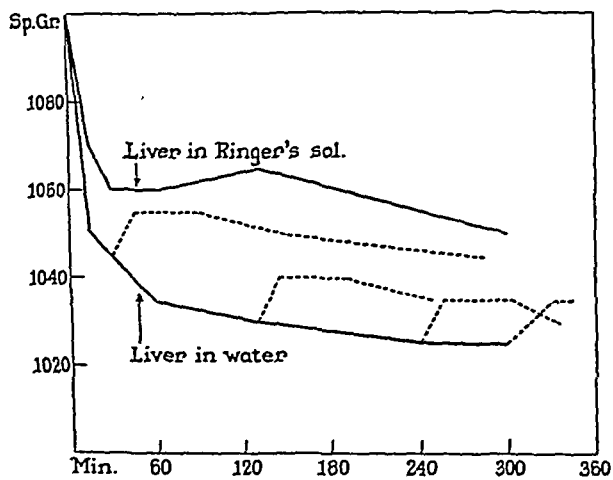


TEXT-FIG. 7. Specific gravity of liver: (*a*) in water; (*b*) in Ringer's solution. The broken lines indicate the changes following transfer of the tissue from water to Ringer's solution.

The same change is found when liver tissue is transferred from water to Ringer's solution (Text-fig. 7). If water immersion is more prolonged, transfer to 0.15 molar solution of sodium chloride or to Ringer's solution causes less elevation of specific gravity, and with increasing periods of water immersion there is diminishing approach to the level represented by continued immersion in the salt solutions (Text-figs. 2, 7, and 8). Nevertheless, after 5 hours of immersion at room temperature (Text-fig. 8) or with continued immersion during 18 hours at a temperature just above freezing, there is considerable increase of specific gravity after transfer to a salt solution.

It is noteworthy that reversal of water intake has occurred when liver tissue previously frozen and thawed has been transferred from water to a 0.3 molar solution of sodium chloride under the conditions that have been described; the osmotic system has not been destroyed.

When liver tissue has been immersed in water during 15 or 30 minutes, the immediate effect of entrance of water is seen, as described above, in a narrow zone at the edge of the tissue where the cytoplasm has become acidophile and foam-like. When liver is transferred from water to the hypertonic salt solution, this change is reversed and after about 30 minutes the affected cells have approached their former condition. The outermost edge of the tissue fragment, where cellular injury has been most advanced, is unchanged but the foam-like appearance has in large part disappeared deeper in and where it persists, the round, clear spaces that give this appearance are much smaller. The basophile zone seen after water immersion is still well defined but here the mitochondria are discrete and well stained though some of them remain round



TEXT-FIG. 8. Specific gravity of liver: (*a*) in water; (*b*) in Ringer's solution. The broken lines indicate the changes after transfer from water to Ringer's solution.

and swollen. In the interior of the tissue, the moderate swelling present after water immersion for the same time has disappeared. (In Text-fig. 2, reversal of swelling is indicated by plus signs.)

When transfer to hypertonic salt solution is made after 1 hour of water immersion, similar diminution of the swelling of cytochondria occurs but restoration of the tissue fragment to the original size is less complete. Immersion in water has caused disappearance of recognizable mitochondria and the basophile material that remains is in the interstices between the round clear spaces of the foam-like cytoplasm. With transfer from water to salt solution, apparent restoration of mitochondrial stain and of clumped basophile substance has been repeatedly noted. The stained mitochondrial bodies are spherical and vary considerably in size. They are usually separated by intervening cytochondria stained by the acid dye. In the interior of the tissue, clumped basophile ma-

terial, lightly stained after 1 hour of water immersion, may have its usual intense stain.

After 2 hours of immersion in water when staining with basic dyes has disappeared and the cytoplasm of liver cells is uniformly foam-like, transfer to salt solution causes the liver cells to shrink, with disappearance of the foam-like character of the cytoplasm so that the cytochondria are now seen as much smaller discrete vesicular bodies.

Effect of Freezing on the Permeability of Liver Tissue to Water

Liver tissue has been frozen by means of liquid carbon dioxide and thawed three times or frozen by a mixture of dry ice and ethylene glycol and maintained frozen during a half hour or longer. In both instances, the tissue has been kept during the procedure in a closed vessel to prevent contact with carbon dioxide. No difference in the results of the two methods has been observed. Freezing does not prevent the entrance of water into the tissue of liver or kidney (Text-figs. 9 and 10) and the changes in specific gravity following immersion in water or in Ringer's solution do not differ conspicuously from those of unfrozen liver under similar conditions.

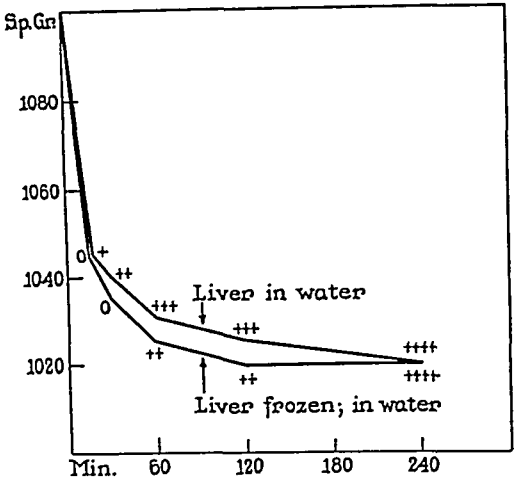
Freezing as such produces immediate changes in the liver cells (Fig. 8). The nuclei are shrunken and their outlines are irregular. The normal delicately formed mitochondria are replaced by scattered particles that take the mitochondria stain. These vary widely in size and some have from 2 to 3 times the usual width of mitochondria.

Within 15 minutes after a piece of liver that has been frozen and thawed is immersed in water, its cells are diminished in size in a zone immediately next the surface with result that liver cell columns are narrow and the spaces between them wide. Sharply defined bodies, deeply acidophile but with the size and shape of mitochondria, are seen in the cells of this peripheral zone. These altered mitochondria may persist during at least 4 hours of immersion. There is no deposition of basophile material a short distance below the surface, such as that seen under similar conditions when liver has not been frozen.

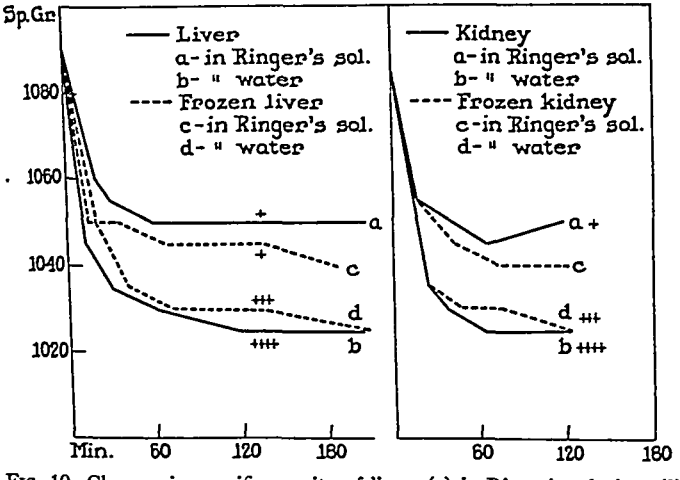
In the interior of the tissue, the coarse round particles that in the frozen liver take the mitochondrial stain have disappeared after 1 hour. The liver cells are diminished in size and the cell columns have become narrower, whereas the spaces between them, occupied by the sinusoids, are widened (Fig. 11; compare with Fig. 10 showing unfrozen liver immersed in water during the same time). Nevertheless, the cytochondria are moderately swollen, vesicular, and spherical. Swelling of the cytochondria continues and in the period from 2 to 4 hours, the cytoplasm becomes foam-like though the cells are still shrunken. The rounded spaces that give the foamy appearance are less uniform in size and shape than those seen after water immersion of unfrozen liver, and large vacuoles occur.

Changes in Kidney Following Immersion in Water and in Ringer's Solution

When slices of kidney tissue are immersed in distilled water, intake of water as measured by specific gravity may occur more rapidly than in liver under the



TEXT-FIG. 9. Specific gravity (*a*) of liver immersed in water; (*b*) of liver frozen and thawed and immersed in water.



TEXT-FIG. 10. Changes in specific gravity of liver: (*a*) in Ringer's solution; (*b*) in water; (*c*) frozen, thawed, and then immersed in Ringer's solution; (*d*) frozen, thawed, and immersed in water. Changes in specific gravity of kidney under corresponding conditions. Changes in cytochondria are indicated by plus signs.

same conditions and equilibrium may be established at a lower level (Text-fig 1), but in other instances liver and kidney have undergone almost identical changes (Text-fig. 10).

Histological examination shows that changes in the kidney following immersion in water reproduces with some modification those which occur in the liver under corresponding conditions. Immediate penetration of water, which is so conspicuous at the cut edges of the liver, is evident only in a very narrow zone at the surface of the slice of kidney and here it proceeds so rapidly that the cytoplasm of the affected cells undergoes almost complete disintegration. In the interior of the tissue, changes proceed more rapidly than in liver. After 30 minutes, the mitochondria are in part deeply stained and rod-like but in part swollen and spherical. Vesicular forms, as in the liver after water immersion, are readily found. With disappearance of mitochondrial stain, the cytoplasm is occupied by round acidophile bodies which vary much in size and may be vesicular. Vacuoles, some large, may give a foam-like appearance to cells of some of the tubules. After 1 hour, this foam-like appearance of the cytoplasm is almost universal, but some staining of mitochondria persists. After 2 hours a few swollen spherical mitochondria are sparsely scattered in some of the cells but, otherwise, the tissue is wholly acidophile. With increasing size of the vacuoles, there is evident disintegration of cytoplasm.

Intake of water by kidney in Ringer's solution, as indicated by changes in specific gravity, has been essentially the same as that of liver. In this solution the mitochondria are preserved for a time, and are well stained as discrete bodies even after 2 hours. They have become spherical and much swollen but in places they are losing the mitochondrial stain to become discrete, round acidophile bodies.

When kidney is frozen and thawed (Text-fig. 10) and then immersed in water, cells of the convoluted tubules, as with previously frozen liver cells, are after 2 hours diminished in size though the cytochondria are swollen, in places so much that their cytoplasm has become foam-like.

RECAPITULATION AND DISCUSSION

Most of the studies that have attempted to define the conditions under which water and other substances enter living tissues have dealth with plant cells, isolated animal cells, such as invertebrate ova, or with erythrocytes. Mammalian tissue have been regarded as an unfavorable object for such experiments. Muscle of frog has been subjected to the action of water or to various solutions more frequently than any other tissue, but the changes that follow indicate that permeability is profoundly influenced by injury to the tissue by the agents that have been used. This opinion is in accord with the studies of Osterhout (5) made with plant cells subjected to the action of various solutions. Overton (6) measured the quantity of water that entered into the muscle of frog when immersed in water and came to the conclusion that it was considerably greater than that held in the cell by osmotic forces. To explain the discrepancy, it has been assumed that part of the water of living tissue is so bound in its rela-

tion to colloids of the cytoplasm that it does not exhibit the usual chemical and physical properties of free water. Most of those who have considered this question in later years (Hill (7); Greenberg, Greenberg, and Cohn (8)) support the conclusion that the water of blood and of muscle, as well as that of such colloids as casein in solution or egg white, has all of the properties of equal quantities of water under usual conditions.

Osmotic swelling of mitochondria has been observed by Fauré-Fremiet (9), Anitschkow (10), Bang and Sjövall (11), Lewis and Lewis (12), and Lazarow (13).

The experiments described here show that changes occurring in tissues immersed in water are dependent not only on the surface properties of the cells themselves, but upon those of particulate bodies within them. The succession of these events indicates that no simple formula will define the action of the forces concerned. The changes that occur at the immediate surface of the excised piece of liver, concurrently with a rapid fall of specific gravity, are determined by direct contact with water whereas those at a deeper level are caused by passage through the tissue of a fluid increasingly modified by constituents of the cytoplasm. The artefacts which result are worthy of consideration because they forecast the changes that occur after a much longer interval in the interior of the tissue and both give information about the structure and function of bodies that constitute a large part of the cytoplasm of cells.

At the edge of the tissue, water promptly removes from the cytoplasm both the basophile material that gives the reactions of ribonucleic acid (2) and the material that gives to the mitochondria their characteristic stain. The cytoplasm of the affected cells now stains only with an acid dye and acquires a foam-like appearance because its cytochondria are swollen by the penetration of water into them. The intensely stained basophile zone at the lower level is doubtless referable to ribonucleic acid or related substances (2). The possibility suggests itself that they are swept inward by the entering water and here find conditions favorable to their deposition in the cytoplasm of the liver cells, but the functional characteristics of liver cells doubtless determine these divergent reactions of adjacent cells. The cytoplasm of some of the basophile cells may assume a foam-like character, like that of cells in the outermost zone with the difference that basophile material occupies the interstices between the clear round spaces within the cytoplasm. Cells in immediate contact with one another show wide difference in their susceptibility to the change and acidophile cells may be wholly or partly surrounded by basophile elements or basophile, by acidophile cells (Fig. 7).

In the cells below the basophile zone, changes are in progress after a few minutes of water immersion that ultimately extend throughout the interior of the block of tissue. Liver cells become swollen and their rounded margins are now conspicuously defined by the acid dye. A succession of changes, presented

diagrammatically in Fig. 14, results in the loss of mitochondrial stain. When the intensity of the stain is diminished following water immersion, some swollen mitochondria have a narrow rim of mitochondrial stain surrounding a clear central space (Fig. 14, *c*). The material that takes the stain appears to lie upon the surface of a body which in its absence stains only with an acid dye (*d*). The mitochondrial material, perhaps like ribonucleic acid, may be adsorbed upon the surface of the body. Changes indicated in the diagram (*e, f*) further suggest that the stainable mitochondrial substance may at one stage of its disintegration collect as a minute droplet on the surface of the acidophile body.

Some swelling of mitochondria occurs before they have lost their peculiar stain but advanced swelling is seen only after they have become acidophile. After 2 hours of immersion in water at a time when the specific gravity of the tissue has reached an equilibrium at its lowermost level, the foam-like appearance of the cytoplasm that results is almost uniformly distributed.

When liver tissue is immersed in a solution of sodium chloride with concentration approximately isotonic with blood plasma (0.15 molar), the changes that occur in water are in large part absent. The specific gravity of the tissue reaches an equilibrium at a level much above that after immersion in water. At the edges of the excised tissue, cells and cytochondria are not swollen as by water but the basophile material here disappears and the mitochondria, which in large part retain their usual size and shape, take the mitochondrial stain with some change from its usual color (violet instead of blue with methylene blue). It seems probable that these bodies have been so altered by the entering fluid that they resist further change. A deeply basophile zone is formed but the cytoplasm of the affected cells does not become foam-like. Nevertheless, changes occur slowly in the interior of the tissue. The mitochondria become moderately swollen, lose their peculiar stain, become acidophile, and finally considerably swollen, but they retain their distinctive appearance longer than during water immersion and the basophile zone persists even after 4 hours of immersion in 0.15 molar solution of sodium chloride. With stronger solutions of sodium chloride (0.3 and 0.4 molar) removal of basophile substance and the material that gives the mitochondrial stain is further delayed.

It is noteworthy that solutions of potassium chloride, which in general produce changes like those of sodium chloride, cause more rapid penetration of water into the tissue as indicated by its specific gravity, equilibrium at a lower level, and ultimately more swelling of cytochondria (see Text-figs. 1 and 4). Changes in the liver that follow immersion in 0.15 molar solution of calcium chloride further emphasize the varied relation of the particulate bodies of the cytoplasm to electrolytes. With the calcium chloride solution, cytochondria immediately exposed to the solution promptly become so swollen that the cytoplasm is foam-like whereas solutions of sodium or of potassium chloride of equal molar concentration cause no swelling of these bodies.

Penetration of water into tissue immersed in Ringer's solution differs little from that in an isotonic solution of sodium chloride. With the former, removal of basophile material (ribonucleic acid and related substances) and of the material that gives the mitochondrial stain occurs more promptly and completely in the outer zone of first contact with the solution and here the cytoplasm of cells becomes foam-like, as with an isotonic solution of calcium chloride. Ringer's solution with one-tenth of its volume replaced by serum, or serum alone, has caused changes in specific gravity like those caused by Ringer's solution (Text-fig. 5).

When tissues that have diminished specific gravity as the result of water immersion are transferred to a solution of sodium chloride (0.15 molar) or to Ringer's solution, the change is reversed and water is lost. The swelling of both cells and cytochondria is reduced, with complete or partial return to their former size. When the tissue is transferred to the salt solution, a short time after its immersion in water specific gravity is restored to a level that it would have attained if it had been for an equal time in the salt solution alone, and return of cells and of cytochondria to their original size is nearly complete; but if water immersion is more prolonged, restoration of specific gravity is increasingly less complete and at the same time, there is but a scanty diminution in the size of swollen cells and cytochondria. Injury caused by the penetration of water has presumably so altered their surface properties that osmotic interchange is permanently altered.

In the experiments described here, freezing has not prevented the entrance of water, as measured by the altered specific gravity of the tissue. This observation is contrary to that of Siebeck (14) who found no taking up of water by frog's kidneys that had been frozen. In the experiments recorded here, entrance of water into pieces of previously frozen liver or kidney immersed in water or in Ringer's solution has not differed essentially from that into unfrozen tissue under similar conditions (Text-figs. 9 and 10). Nevertheless the cytological changes have differed widely in frozen and unfrozen tissue following immersion. Osmotic swelling of cells and of cytochondria has not gone on in parallel; the liver cells have been conspicuously shrunken whereas the cytochondria have been swollen. Though liver cell columns are diminished in width, there is a corresponding increase in the spaces carrying sinusoidal vessels (Fig. 11). Perhaps the interstitial tissue of the liver has taken in a quantity of water sufficient to bring the specific gravity to a level approximately equal to that of unfrozen liver similarly immersed.

CONCLUSIONS

The cytoplasm of cells of the liver and of the kidney is in large part occupied by bodies which respond to the water content of these cells and are modified by dissolved substances in the surrounding fluid or by physical change such as freezing.

These bodies, in part mitochondria but designated more broadly cytochondria, constitute an osmotic system within the cytoplasm of cells.

When the specific gravity of liver or kidney tissue is used as an index of changes in the water content of tissue, swelling of cytochondria in general follows the intake of water but this relation may be modified by a variety of conditions.

When liver that has been frozen and thawed is immersed in water, cytochondria become swollen though the containing cells diminish in size.

Solutions of sodium and of potassium chloride isotonic with blood plasma cause delayed swelling of cells and cytochondria, greater with the potassium salt; solutions of calcium chloride of equal molar concentration cause immediate swelling of cells and cytochondria.

The basophile material of the cytoplasm (ribonucleic acid and related substances) and the material that gives to mitochondria their characteristic stain are removed by immersion in water but their disappearance is retarded by isotonic solutions of sodium or of potassium chloride and further delayed by hypertonic solutions.

When the intensity of staining reactions is diminished by the partial loss of basophile substance or of the distinctive mitochondrial material, these are found at the surfaces of the cytoplasmic bodies, held perhaps by adsorption.

When water, isotonic solutions of sodium chloride, or Ringer's solution comes into contact with immersed liver, they remove basophile and mitochondrial material from a superficial zone and substances with similar staining reactions appear in the cytoplasm of cells at a deeper level.

Osmotic changes in the cytoplasmic bodies may be reversible. When liver tissue which has been for a short time immersed in water is transferred to a solution that is approximately isotonic in relation to blood plasma, swollen cytochondria return in part or completely to their former size; but with continued immersion in water, this reversibility becomes increasingly less complete.

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EXPLANATION OF PLATES

These photographs were made by Mr. J. A. Carlile.

Tissues have been fixed in lanthanum acetate and formalin and stained with methylene blue, azure II, and rose bengal.

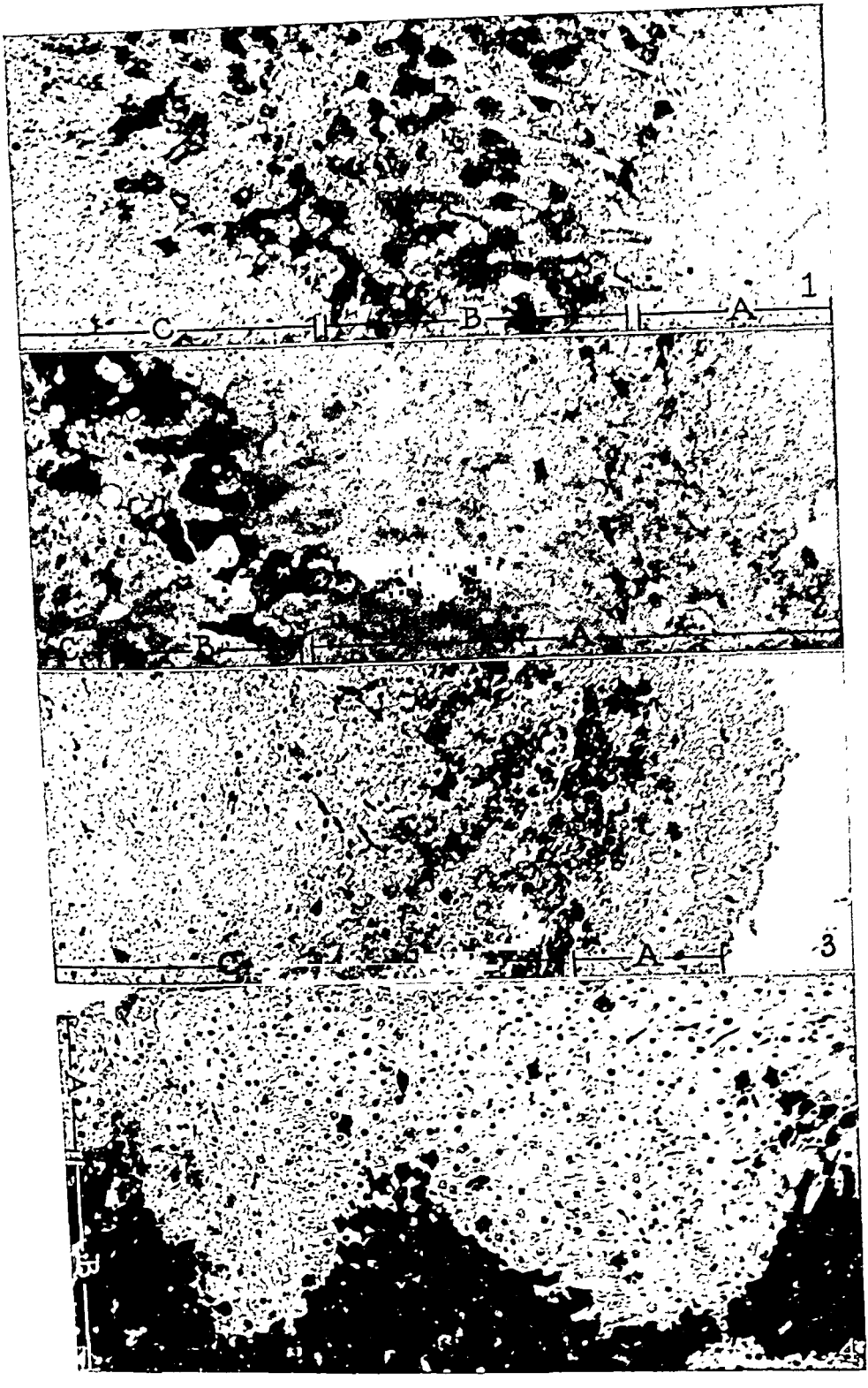
PLATE 6

FIG. 1. Showing changes at the surface of a piece of liver after immersion in water during 15 minutes. In an external zone (*A*) basophile material is no longer visible and the cytoplasm of the cells is foam-like. In a deeper zone (*B*) there is intensely stained basophile material in the cytoplasm of the cells. The changes that have occurred in a third deeper zone (*C*) are shown in Fig. 13. × 212.

FIG. 2. Showing changes at the surface of a slice of liver after immersion in water during 1 hour. In the outermost (acidophile) zone (*A*) the following changes have occurred: cells immediately below the surface are partially disintegrated; cells immediately next them further in are shrunken (and containing altered mitochondria, not shown); cells in the greater part of the zone are swollen (with foam-like cytoplasm as in Fig. 6). Below the acidophile zone (*A*) is an irregularly disposed basophile zone (*B*) and (*C*) representing the interior of the slice of tissue. × 212.

FIG. 3. Showing changes at the surface of a slice of liver after immersion in 0.15 molar solution of sodium chloride during 4 hours. They resemble those after immersion in water (see Figs. 1 and 2) but higher magnification shows that cells and cytochondria in the outermost zone are not swollen (see Fig. 9). For description of (*A*), (*B*), and (*C*) see Fig. 2. × 212.

FIG. 4. Liver tissue showing a sharply defined acidophile (*A*) and deeply basophile zone (*B*) after immersion in Ringer's solution during 2 hours. × 212.



(Opie: Osmotic system within cytoplasm of cells)

PLATE 7

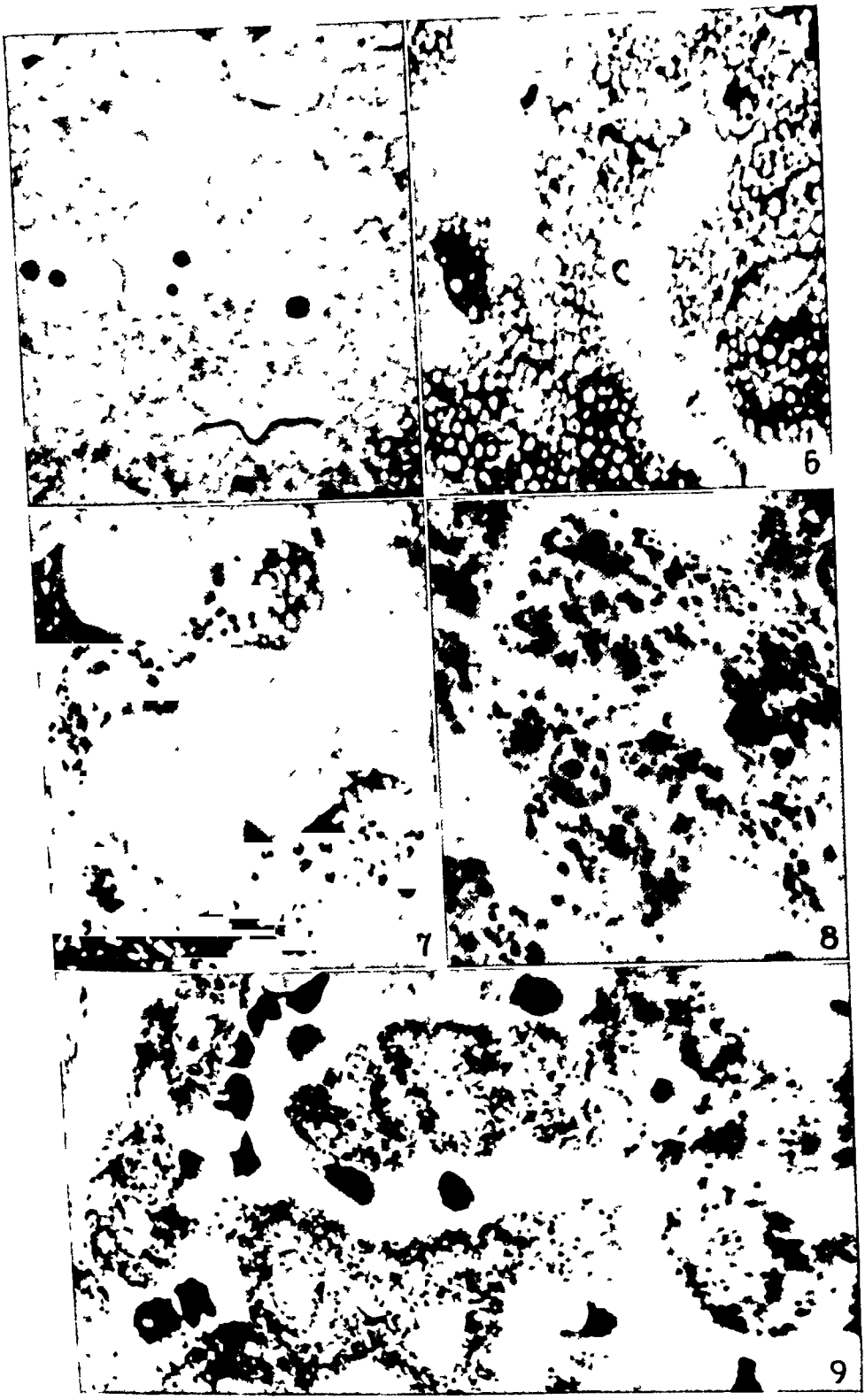
FIG. 5. Showing swollen cells containing swollen vesicular cytochondria in liver cells immersed in water during 1 hour. Three nuclei with nucleoli are seen. × 1500.

FIG. 6. Showing cells with foam-like cytoplasm from liver tissue immersed in water during 1 hour. × 1500.

FIG. 7. Showing acidophile and basophile cells with foam-like cytoplasm at junction of acidophile and basophile zones from liver of guinea pig after immersion in water during 30 minutes. × 1460.

FIG. 8. Showing in frozen and thawed liver tissue diminution of normally stained mitochondria and globular bodies that take the mitochondrial stain. The presence of the nucleus of one cell is well defined. × 1460.

FIG. 9. Liver tissue immersed in 0.15 molar solution of sodium chloride during 3 hours, showing mitochondria with altered stain (violet with methylene blue) in shrunken cells with no basophilia. × 1460.



(Opie Osmotic system within cytoplasm of cells)

Plate 8

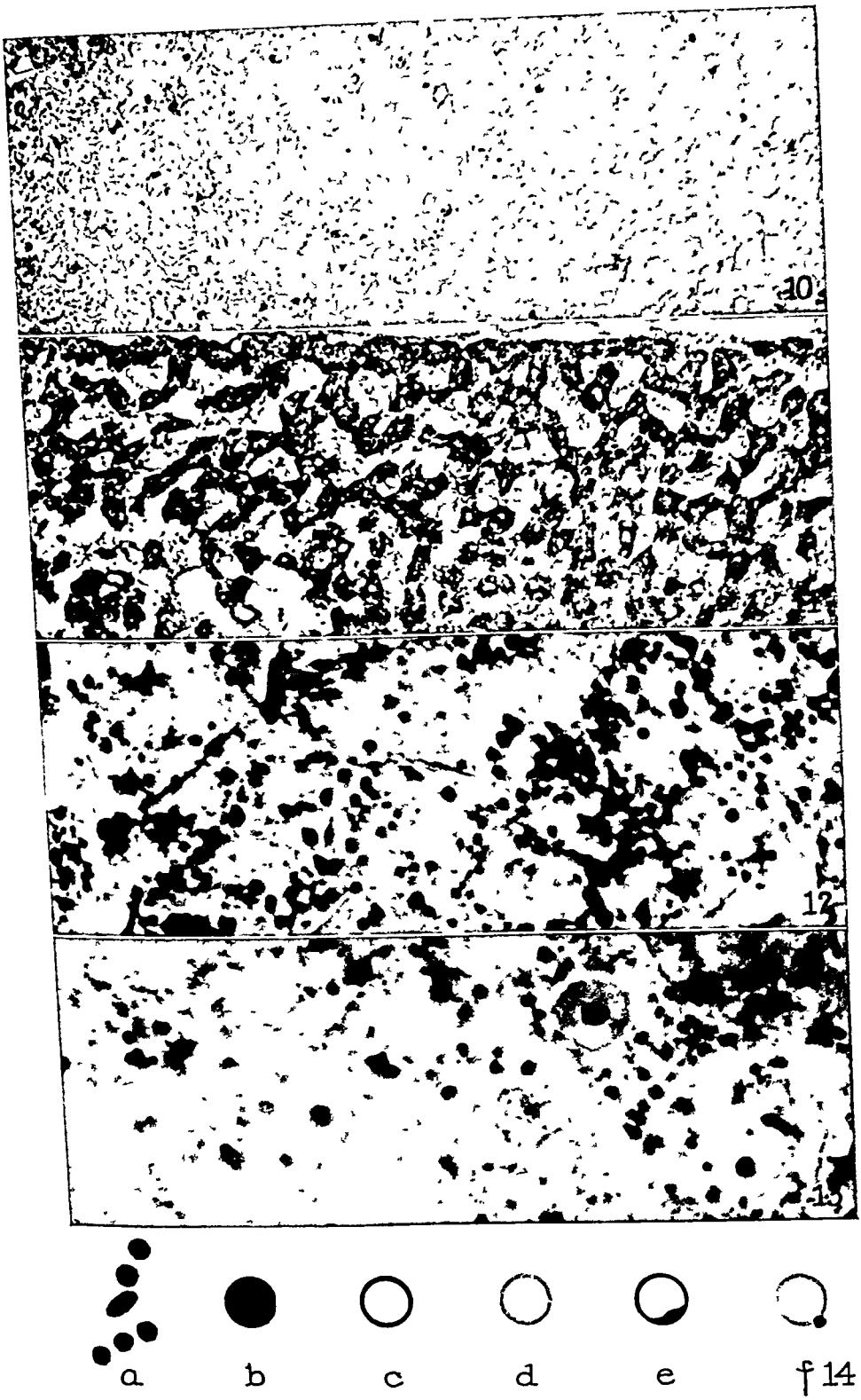
Fig. 10. Showing swelling of liver cells after immersion in water during 2 hours. (For comparison with Fig. 11.) × 260.

Fig. 11. Showing shrunken liver cells with wide spaces between the columns in hepatic tissue frozen and thawed and then immersed in water during 2 hours. × 260.

Fig. 12. Showing swollen mitochondria and acidophile cytochondria (with lighter color in photograph) in guinea pig liver tissue after immersion in water during 30 minutes. Nuclei are poorly defined. × 1460.

Fig. 13. Showing swelling of some mitochondria in rat liver which has been immersed in water during 15 minutes. A greater number of these elements have lost the mitochondrial stain and remain as acidophile bodies (those with lighter color in the photograph). The area shown is from the innermost zone (*C*) of Fig. 1. Nuclei with nucleoli are well defined. × 1500.

Fig. 14. Diagram representing changes in mitochondria caused by water (see Fig. 12 and 13; though not well defined in the photographs): (*a*), mitochondria; (*b*), swollen mitochondrium; (*c*), vesicular mitochondrium; (*d*), vesicular acidophile body; (*e*), vesicular mitochondrium; (*f*), vesicular acidophile body and minute spherical particle with mitochondrial stain.



(Opie Osmotic system within cytoplasm of cells)

THE INACTIVATION OF BIOLOGICALLY ACTIVE PROTEINS, AND THE VIRUS OF WESTERN EQUINE ENCEPHALOMYELITIS BY PERIODIC ACID

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When the toxic somatic antigens of *Shigella paradysenteriae* are treated with periodic acid a rapid loss of serological activity occurs accompanied by a marked diminution in the toxicity of the lipocarbohydrate-protein complex (1). The chemical reaction is probably not confined to an oxidation of the carbohydrate moiety of the antigen, and may well involve the protein component, for there is reason to believe that the toxin is an integral part of the latter and is not associated with the carbohydrate constituent (2).

Beyond the fact that periodic acid will oxidize certain amino acids (3), little is known of its action upon native proteins. Nor is it known whether proteins endowed with biologically active properties can be rendered inactive by the reagent under physiological conditions of pH. Recently it was pointed out that the virus receptor group of fowl erythrocytes is destroyed by periodate, and hence might be carbohydrate in nature (4). That this deduction may not be valid, however, will be seen from the following, for it will be shown that certain well defined proteins undergo radical chemical and biological changes when subjected to the action of the reagent. These changes are such that they destroy not only the biological activity of the protein, but in the one instance studied this change was accompanied by a profound alteration in absorption spectrum. In addition, it will be demonstrated that the virus of Western equine encephalomyelitis which, for lack of better evidence, has come to be regarded as protein in nature (5) is rapidly and completely inactivated by the action of the reagent in neutral solution.

THE ACTION OF PERIODIC ACID ON PROTEINS

Materials and Methods.—The crystalline ribonuclease used in these studies was kindly supplied by Dr. M. McCarty. The activity of the enzyme before and after treatment with periodic acid was determined by the procedure of Kunitz (6).

The Type III pneumococcus antibody globulin was prepared by the dissociation of an immune precipitate as described later. Total precipitable nitrogen was estimated by micro-Kjeldahl. The decrease in activity of the immune globulin on treatment with lithium periodate was determined photometrically using the homologous specific polysaccharide as

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precipitinogen (7). 0.1 M lithium periodate in 0.02 M lithium phosphate was prepared by dissolving 2.28 gm. $HIO_4 \cdot 2H_2O$, and 10 ml. of 0.2 M H_3PO_4 in 50 ml. H_2O followed by the addition of 20 ml. of 1.0 N LiOH and diluting to 100 ml. in a volumetric flask.

Immune sera to the native and inactivated immune globulins were obtained from two groups of rabbits which had received intravenous injections of the two proteins. 4.0 mg. of the respective proteins were injected daily for 6 days, followed by an 8 day rest period. The course of injections was repeated, and the sera collected 8 days after the last injection.

The Inactivation of Ribonuclease by Periodic Acid

In order to study the effect of periodic acid on crystalline ribonuclease, a solution of the latter was subjected to action of periodate at pH 5.1 and at

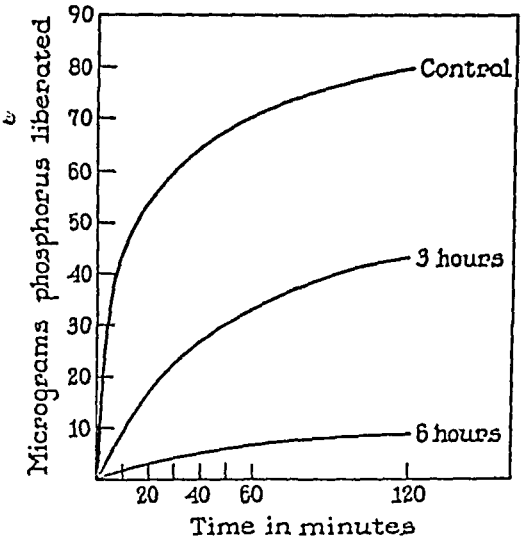

FIG. 1. Liberation of soluble phosphorus from ribonucleic acid by ribonuclease before and after treatment with periodic acid.

25°C. for varying intervals of time. The activity of the enzyme solution was determined and compared with that of untreated enzyme.

A stock solution of ribonucleic acid containing 0.5 mg. phosphorus per ml. in 0.1 M acetate buffer was prepared. A solution of ribonuclease containing 0.10 mg. protein nitrogen per ml. was likewise prepared in 0.01 M acetate buffer and stored at 0°C. The periodic acid-buffer solution was made by mixing equal parts of 0.2 M periodic acid and 0.75 M sodium acetate. The pH of the solution was 5.1.

To study the effect of periodic acid on the enzyme, 5.0 ml. of the latter was mixed with 5.0 ml. of reagent at 25°C. and the mixture allowed to stand. At the end of 3, 6, and 24 hours 2.0 ml. of solution was removed, transferred to a cellophane bag containing 0.3 ml. of 50 per cent glucose, and dialyzed in a rocking device for 4 hours against a flow of distilled water. The contents of the bag were transferred quantitatively to a 10 ml. volumetric flask, and the activity of the enzyme determined. 5.0 ml. of enzyme, now containing 0.01 mg. protein nitrogen per ml., was mixed with 5.0 ml. of stock ribonucleic acid solution, and the liberated phosphorus determined at the end of 30, 60, and 120 minutes. The results are presented graphically in Fig. 1. For purposes of comparison this figure includes the activity curve of the untreated ribonuclease.

From the results presented in Fig. 1 it is apparent that treatment of the enzyme at pH 5.1 with periodic acid brings about a gradual loss of biological activity, and this change is not rapid. Inactivation is apparently complete after contact with the reagent for 24 hours, for it is not possible to demonstrate the liberation of phosphorus from ribonucleic acid by the enzyme treated for this period of time. The nature of the chemical change in the enzyme molecule brought about by the oxidant is not known, but that it is profound is evident from the fact that a complete loss of biological activity occurs.

The Inactivation of Type III Pneumococcus Antibody by Periodic Acid

Preparation of Antibody.—A second protein was chosen for study in order to determine whether biological activity of an entirely different nature would be affected by periodic acid. Type III pneumococcus immune horse globulin was selected, because it can be had in relative abundance.

The purified antibody was prepared by dissociating an immune precipitate (8) obtained from the interaction of 3 liters of antipneumococcus horse serum Type III, and the appropriate quantity of homologous type specific polysaccharide. The serum was diluted to 13 liters with 0.9 per cent NaCl; 0.25 gm. of Type III polysaccharide dissolved in 1 liter of saline solution at 5°C. was added. This quantity sufficed to carry down the major part of the antibody, but still left a slight excess in the supernate.

After 2 hours the immune precipitate was spun at 0°C., washed five times with cold saline solution, and suspended in 400 ml. of 25 per cent ammonium sulfate. The solution was stirred and warmed to 37°C. for an hour, spun, and then diluted to 50 per cent saturation with saturated ammonium sulfate. The dissociated antibody which precipitated was collected by centrifugation, and twice reprecipitated by half saturation with ammonium sulfate. The antibody was then dissolved in water and dialyzed free of SO_4^{--} against 0.9 per cent NaCl. Small amounts of precipitate which separated during the dialysis were centrifuged and discarded. From 25 to 38 per cent of the protein originally present in the immune precipitate was recovered by this procedure. The dissociated antibody prepared as described above varied in its specific precipitable nitrogen content from 65 to 82 per cent.

Inactivation of Pneumococcus Antibody by Periodic Acid.—A solution of Type III antibody containing 1.0 mg. nitrogen per ml. was treated with an equal volume of 0.05 M $LiIO_4$ at pH 7.2. At varying time intervals 0.5 ml. samples were removed, added to 0.2 ml. 50 per cent glucose in a 5.0 ml. volumetric flask, and diluted to the mark with 0.9 per cent NaCl. Samples of 0.40, 0.20, and 0.10 ml. were removed, placed in calibrated tubes, diluted in each instance to exactly 1.00 ml. Then 1.00 ml. of a dilution of Type III polysaccharide containing 10 micrograms of carbohydrate was added to each tube. After 30 minutes the turbidity was measured in a phototurbidometer, and the result recorded (Fig. 2). When the classical quantitative precipitin reaction was carried out on the various treated antibody solutions, the results were essentially the same as those obtained photometrically. It can be seen that the precipitability of the immune globulin is rapidly impaired by contact with the reagent, and is entirely destroyed when the globulin remains in contact with the periodate for 24 hours (not shown on graph).

Change in the Absorption Spectrum of Immune Globulin Treated with Lithium Periodate at pH 7.2.—A solution of Type III pneumococcus anticarbohydrate-immune globulin containing 20.0 mg. protein per ml. was treated with an equal volume of 0.05 M $LiIO_4$ at pH 7.2 for 24 hours. The reagent was removed by rapid dialysis against physiological salt solution, and diluted to a concentration of 1.0 mg. protein per ml. in 0.02 M phosphate buffer at pH 7.6. The

absorption spectrum of this material was measured and compared with that of untreated immune globulin of the same concentration in the same buffer. The absorption spectrum is shown in Fig. 3, where *a* represents the absorption spectrum of the native globulin, and *b* that of the globulin after treatment with lithium periodate.

It is evident that a marked change in the absorption spectrum has taken place. Not only is there an increase in absorption at the maximum as well as a slight shift to a shorter wave length, but there is a relatively greater increase in the minimum absorption coefficient. The reason for this is not as yet known, but it should be pointed out that Gates (9) observed a similar shift in the absorption spectrum of crystalline pepsin after irradiation with ultraviolet light. Furthermore, Hicks and Holden (10) have measured the absorption spectra of proteins denatured by alkali and by alcohol, and observed an

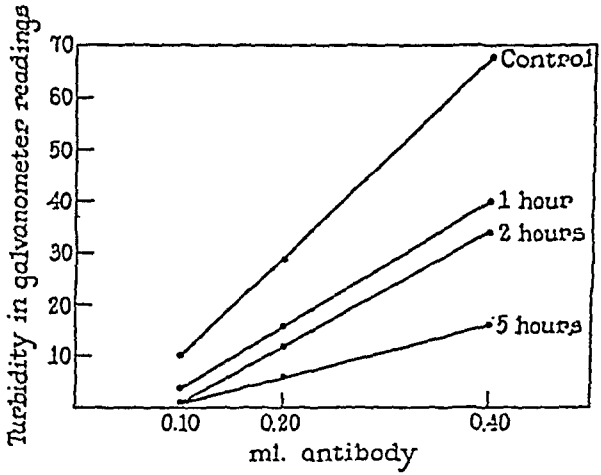

FIG. 2. Inactivation of Type III pneumococcus antibody globulin by lithium periodate.

increased absorption through the entire range of wave lengths. Whether the change in absorption spectrum of the immune globulin which follows treatment with periodate represents true denaturation comparable to that brought about by alcohol or alkali cannot yet be said with certainty. In this connection, however, it should be pointed out that after such treatment, a portion of protein, but by no means all, will precipitate in the presence of 0.9 per cent sodium chloride when the pH of the solution is shifted from neutrality to 4.8.

Specificity of Antisera Prepared by Immunization with Inactivated Immune Globulin.—That a marked chemical change has taken place when immune globulin is treated with periodate at pH 7.2 is apparent from the foregoing experiments. Whether this change is sufficiently great to affect the antigenicity of the altered protein was not known.

A group of rabbits was injected with daily doses of 4 mg. of the active protein for 6 consecutive days, following a rest period of 8 days; the course of injection was repeated, and 8 days following the last injection, the animals were bled. A second group of animals was likewise

immunized with untreated immune globulin. The sera were then tested for homologous precipitins (Table I) and heterologous precipitins (Table II).

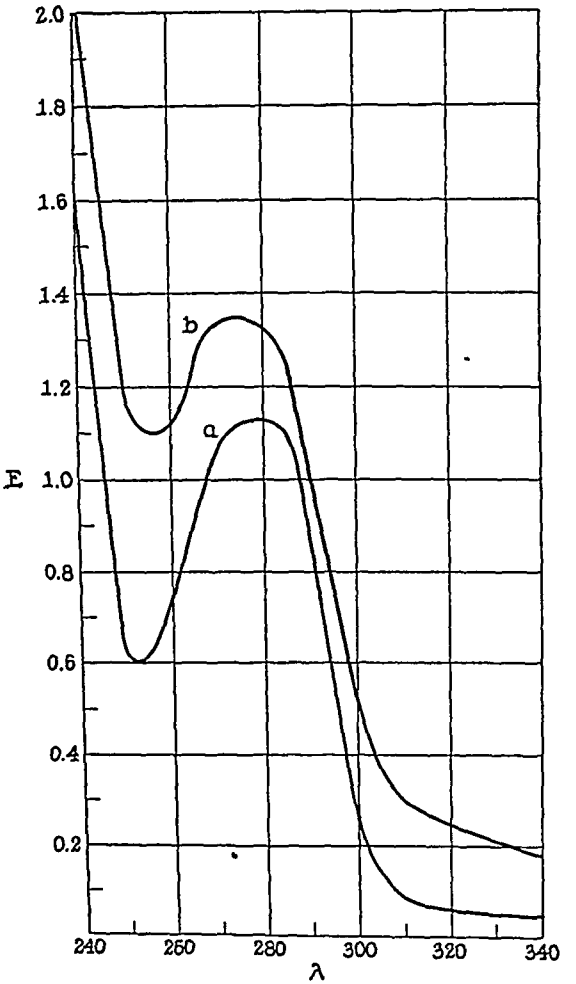

FIG. 3. Absorption spectra of Type III pneumococcus antibody globulin before and after treatment with lithium periodate.

Immune globulin, treated with lithium periodate for a period of 24 hours and completely inactivated, is fully antigenic, as can be seen from Table I. Furthermore, when the inactivated protein is tested in the antiserum prepared by injecting rabbits with untreated and fully active immune globulin, marked precipitation occurs (Table II). If the respective sera are absorbed with the heterologous antigen, and then tested with the homologous protein, no reaction occurs indicating that the antibody is completely removed (Table III) by the

heterologous protein. From the results of these experiments it can be concluded that although destruction of biological activity takes place when the

TABLE I

Homologous Precipitin Reactions of Sera of Rabbits Immunized with Type III Pneumococcus Antibody Globulin, before and after Treatment with Lithium Periodate

Rabbit No.	Immunized with*	Test antigen	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
1	G_{III}	G_{III}	+++	++	+±	+
2			++++	++±	+±	+
3	G_{III} (HIO_4)	G_{III} (HIO_4)	+++±	++±	++	+
4			+++	++	+±	+

* In Tables I and II, G_{III} refers to untreated Type III pneumococcus immune globulin; G_{III} (HIO_4) to the globulin treated 24 hours with $LiIO_4$.

TABLE II

Heterologous Precipitin Reactions of Sera of Rabbits Immunized with Type III Pneumococcus Antibody Globulin, before and after Treatment with Lithium Periodate

Rabbit No.	Immunized with	Test antigen	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
2	G_{III}	G_{III} (HIO_4)	+++±	++±	+±	+
3	G_{III} (HIO_4)	G_{III}	+++	++	+	±

TABLE III

Cross-Precipitin Reactions in Absorbed Rabbit Sera

Serum of rabbit immunized with:	Serum absorbed with	Test antigen used	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
G_{III}	Unabsorbed	G_{III}	+++±	++±	+±	+
	G_{III} (HIO_4)	"	0	0	0	0
	"	G_{III} (HIO_4)	0	0	0	0
G_{III} (HIO_4)	Unabsorbed	G_{III} (HIO_4)	+++	++	+	±
	G_{III}	"	0	0	0	0
	"	G_{III}	0	0	0	0

immune globulin is treated with periodate, neither the capacity of the protein to incite specific immune bodies is destroyed, nor is the species specificity of the protein lost. If treatment of the immune globulin with periodate is accompanied by denaturation, it is difficult to understand why the specificity of the antibody elicited by the treated protein is not altered. The consensus of

opinion is that the denaturation of proteins endows them with a new and changed specificity (11).

THE ACTION OF PERIODIC ACID ON A VIRUS

The action of potassium and lithium periodate on the virus of Western equine encephalomyelitis was then investigated.

Materials and Methods.—The Rockefeller Institute strain of the virus used in these studies was stored frozen, at about −70°C., in the form of 20 per cent mouse brain in 50 per cent normal rabbit serum. The LD_{50} of the thawed material as tested in the Rockefeller Institute strain of albino mice, was about $10^{-8.5}$. Before determining the effect of periodate upon the virus, the latter was first diluted with an equal volume of 10 per cent normal rabbit serum, the suspension was then centrifuged at 10,000 R.P.M. for 30 minutes, and the supernate, containing the virus, was employed. To 1 ml. of the supernate virus were added 2 ml. of 0.01 M phosphate buffer at pH 7.0, 1 ml. of 0.05 M KIO_4 solution, and 5.5 ml. of 0.4 per cent NaCl solution. After a period of contact varying from 7 minutes to 3 hours at 37°C. (depending on the particular experiment of those to be described later), 0.5 ml. of 50 per cent dextrose solution was added to decompose the periodate. Thus an additional tenfold dilution of virus was obtained. The mixture was then dialyzed under sterile conditions in a mechanical rocker for 4 hours against a constant flow of 0.01 M phosphate buffer at pH 7.0. The materials were removed and titrated for virus activity by the intracerebral inoculation of mice, making dilutions in 10 per cent normal rabbit serum (12). For each experiment the appropriate controls were included as well as a titration of the same untreated virus sample as employed in the test (Table IV). In later tests, the lithium salt of periodic acid, because of greater solubility, was substituted for the corresponding potassium salt. When the lithium salt was employed certain other modifications were introduced; *e.g.*, 1 ml. of 20 per cent virus was added to 1 ml. of 0.05 M or 0.1 M $LiIO_4$ in 0.02 M lithium phosphate buffer at pH 7.2. After contact for varying periods of time at 37°C., 0.2 ml. of 50 per cent dextrose solution was added. The procedure of dialysis, dilution of dialyzed materials, and mouse test was otherwise unchanged.

The complement-fixation tests as here reported were performed after the manner described by Casals (13). The virus exposed to the periodate for 10, 30, and 60 minutes comprised the antigen. The latter was serially diluted up to 1/64; the serum from 0 to 1/128. Known standard equine virus antisera and those against St. Louis and Japanese B encephalitis viruses were tested as controls along with the serum from mice immunized as described in the next paragraph (13).

To test for the presence of an immunity developed to the periodate-treated virus, mice were injected intraperitoneally with material inactivated for 10, 30, and 60 minutes; 120 to 210 mice were included in each test. Six immunizing doses of 0.25 ml. each were given, in two series of three consecutive daily injections with a 5 day interval between the series. To test for any immunity that might have been developed, 11 days after the last dose, dilutions of 10^{-3}, 10^{-5}, 10^{-6}, and 10^{-7} of virus were inoculated intracerebrally into 6 or more mice for each dilution. Similar injections were given to uninoculated animals.

For the detection of complement-fixation antibody, serum from these mice was collected. For determination of virus-neutralization antibody, serum was obtained from rabbits which had received repeated doses of 3 to 10 ml. of virus intraperitoneally over a period of 29 days. The virus employed was a sample which had been in contact with the periodate for 10 and for 30 minutes. The neutralization and complement-fixation tests for these antibodies were performed in the manner already described (12, 13).

Effect of Periodate on Virus Titer.—A prompt and marked inactivation of

the virus of Western equine encephalitis occurred after exposure to potassium or lithium periodate. Table IV, which represents but a single test, shows that while some inactivation of virus was demonstrable within 10 minutes contact with a 0.05 M solution of the reagent more than 25 million doses were inactivated after a period of 2 hours. These observations were confirmed by the results of other tests in which it was found that within 7 to 10 minutes from 100,000 to 1,000,000 doses of virus were rendered inactive by periodate of 0.1 M concentration. To summarize the results of a number of experiments, it would appear that both 0.05 M potassium and 0.05 or 0.1 M lithium periodate could inactivate an appreciable amount of virus within a few minutes. The effect, however, increased with the duration of the time of contact so that most of the

TABLE IV

Showing Inhibition of the Virus of Western Equine Encephalitis by 0.05 M $LiIO_4$, and the Influence of Time of Contact upon the Reaction

Tests					Controls		
Virus dilution	Time of contact with $LiIO_4$ at 37°C.				Virus dilution	1 hr.	2 hrs.
	10 min.	30 min.	1 hr.	2 hrs.			
10^{-1}	5/5*	5/5	3/5	2/5	10^{-7}	5/5	5/5
10^{-2}	5/5	3/5	1/5	0/5	10^{-8}	5/5	4/5
10^{-3}	5/5	2/5	0/5	0/5	10^{-9}	1/5	0/5
10^{-4}	3/5	0/5	0/5	0/5	10^{-10}	0/5	0/5
LD_{50}	$\geqslant$4.2‡	2.5	1.3	<1.0		8.6	8.4

* Number of mice succumbed to virus infection over number of mice used.

‡ Reciprocal of log of dilution.

virus added was made ineffective within ½ to 1 hour, and all the virus that could possibly be used within the limits of the tests, *i.e.* over 300 million lethal doses, was rendered inert within 2, 2½, or 3 hours.

The reduction in titer of the virus by means of periodate was a function not only of time of contact (Table IV) but also of temperature. Thus, when the test was carried out at 37°C., as compared with 4°C., a greater degree of inactivation ensued at the higher temperature, greater by at least 20 per cent. It was also found that the pH of the mixtures, which is varied but little before and after dialysis, was not a factor in the reduction of the titer of the virus. For example, electrometric determinations revealed that the pH of the mixtures usually ranged from 6.8 to 7.1, either at the time of mixing or before their inoculation into animals.

Although the data are not given it was shown that the periodate ion rather than potassium or lithium ion was responsible for the inactivation of the virus. Also, the reduction in titer of the virus was the result of the action of the

periodate itself, not that of a secondary substance which might have developed from the interaction of the component materials used in the test.

Effect of Periodate on the Antigenicity of the Virus.—The problem of the effect which periodate exerted on the antigenicity of the virus was studied from two angles. In the first tests, attempts were made to immunize mice to a test dose of virus by injecting periodate-treated virus repeatedly. In the second series of experiments the serum of such treated animals was examined for the presence of induced complement-fixing antibody. In addition, a search was undertaken for virus-neutralizing antibody in the sera of rabbits injected repeatedly with periodate-treated virus.

In summary, the treated virus failed in all instances to induce either active or passive immunity, even when exposure to the reagent was as short a time as 10 minutes. Such material also did not bring about the development of serum antibody, either complement-fixing or virus-neutralizing. To be sure, virus treated with the periodate for 10 or 30 minutes and used in these tests as antigen, contained a certain amount of active virus and a degree of immune response was at times noted. The response, however, was no greater than that expected from the immunogenic action of the small amount of residual virus present in the inocula. The relationship of the amount of virus used to the degree of immunity induced has already been established (14, 15). To conclude, just as the virus of Western equine encephalitis was inactivated rapidly by exposure to the action of periodate, so was its antigenicity completely destroyed, even within as short a time as 10 minutes, under the experimental conditions here employed.

DISCUSSION

Periodic acid is an oxidizing agent which has been extensively employed in the study of the structure of carbohydrate and steroid derivatives. Its value can be attributed to the fact that in general its oxidizing action is selective for it severs linkages only between those carbon atoms bearing adjacent hydroxyl, or hydroxyl and amino groups. We became interested in the reagent when it was observed that the somatic antigens of dysentery bacilli were readily detoxified by periodate. Since the toxic component of these microorganisms appears to be intimately associated with the protein and not the carbohydrate portion of the antigenic complex, it was reasonable to assume that the former must be altered chemically by the reagent. A perusal of the literature revealed little concerning the action of periodate upon proteins. Desnuelle and Antonin (16) observed that when several proteins, including egg albumin, were treated with sodium periodate there was no liberation of formaldehyde and assumed that the reagent induced only an insignificant chemical change in these proteins. That certain amino acids, such as serine and threonine, are quantitatively oxidized is of course well known.

In the present investigation it has been demonstrated that when a subtle biological function is involved, this latter may be readily impaired when the protein concerned is subjected to the action of periodate at physiological values of hydrogen ion concentration. Not only was the biological activity of two widely divergent proteins, an enzyme and an antibody, lost, but the pathogenicity of a virus was destroyed. It is of interest that although the serological specificity of Type III pneumococcus immune globulin was rapidly impaired, and finally destroyed by periodate, the capacity of treated immune globulin to elicit antibody in experimental animals was not. This is in direct contrast to the results observed with Western equine encephalomyelitis virus, where contact with the reagent, even for a very brief time interval destroyed, under the experimental procedures used, its infectivity and its ability to elicit specific antibody.

The nature of the chemical action of periodic acid upon the proteins studied cannot at the present time be elucidated. No intensive attempts have been made as yet to disclose the mechanism of the action of the reagent upon these substances beyond the demonstration that a change in the absorption spectrum of immune globulin accompanies contact with the reagent. Whether certain terminal amino acids such as serine or threonine are oxidized cannot yet be said. That certain proteins are readily inactivated by the reagent is evident from the foregoing experimental data. One should be reserved in the assumption that because the biological function of an unknown substance is impaired by contact with periodate, the material should be classified as a carbohydrate.

SUMMARY

The action of periodic acid on two biologically active proteins, crystalline ribonuclease and pneumococcus Type III immune globulin, and on the virus of Western equine encephalomyelitis has been studied. The biological activity of the two proteins and the pathogenic action of the virus were destroyed by the reagent; the specific antigenicity of the immune globulin was retained, however, but that of the equine virus was lost. The bearing of these reactions on the chemical alteration of the respective substances has been discussed.

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STUDIES
FROM
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Announcement

Volumes 131 and 132 of the *Studies from The Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

A STUDY OF NERVE PHYSIOLOGY

The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1060 pages with 480 illustrations.

In order that the volumes may be available to those who do not receive them as part of their subscriptions to the *Studies*, extra copies have been printed which are purchasable at the regular price of $2.00 per volume. The two volumes will only be sold together. They can be obtained from the Publication Service, The Rockefeller Institute for Medical Research, York Avenue and 66th Street, New York 21, N.Y., at a price of $4.00, payable in advance.

PROTEIN METABOLISM AND EXCHANGE AS INFLUENCED BY CONSTRICTION OF THE VENA CAVA

Experimental Ascites an Internal Plasmapheresis: Sodium Chloride and Protein Intake Predominant Factors

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Workers in this laboratory have long been interested in the production of plasma proteins and the fluid exchange which goes on between the circulating plasma proteins and various body tissues. Ascites comes into consideration in such relation and the experimental method of vena cava constriction was used to study the body protein exchange due to ascites. As the experiments continued it was obvious that this method presented an admirable technique for the study of ascites and some of the related medical problems. Furthermore, the ascites was not complicated by the presence of extensive liver pathology and was due to venous stasis in the portal area. Ascites was produced in the dog by placing a constricting aluminum band on the inferior vena cava between the diaphragm and the right auricle.

This method of vena cava constriction was used by one of us (G. H. W.) in 1909–10 to produce ascites. The partial obstruction was caused by sutures put in the vena cava to produce narrowing of the lumen. This condition would last many weeks and then the sutures would cut through with relief of the obstruction and complete absorption of large amounts of ascitic fluid in a matter of hours. In these experiments there was no significant liver pathology remaining after relief of the obstruction. As these earlier experiments were incidental to a study of liver abnormalities (17) they were soon discontinued.

The tables below show clearly that vena cava obstruction and the resultant ascites may produce a considerable drain on protein reserve stores and a hypoproteinemia. As the ascitic fluid is removed at frequent intervals, there results a continuing *internal plasmapheresis*. The term *plasmapheresis* as used here means the removal of whole blood with subsequent replacement of the red cells suspended in a salt solution. As the procedure is used day by day one observes an hypoproteinemia which can be maintained for months at an even level. This technique has been used extensively to study the production of new plasma proteins as influenced by diet factors (10, 12–14).

Constriction of the vena cava between the right auricle and the diaphragm is not a new procedure, but previous investigators have used the method for entirely different purposes. In 1903, Bolton (2) attempted a study of "cardiac dropsy," artificially induced in cats by constriction of the pericardium which interfered with the diastolic filling of the heart. The method, however, proved unsatisfactory for a large series of experiments because so many of the animals died from the operation or recovered perfectly without developing dropsy. Bolton then (3, 5) constricted the inferior vena cava and found that complete occlusion led to death in a few hours. The vein had to be constricted three-fifths of its diameter to produce any dropsy. In another publication (4) he stated that ascites due to incomplete obstruction gradually increased to a certain point of time—persisting for about 2 to 3 months—when it disappeared with the complete establishment of collateral circulation. In this same paper he reported an experiment on a monkey in which the inferior vena cava was constricted to one-half its diameter and the animal killed 1 year later. At autopsy he found the vein occluded completely by scar tissue, and the liver enlarged with the appearance of the human "hob-nail" liver. Microscopically, he found no inflammatory changes, but areas of central degeneration which blotted out entire lobules in many places. The portal canals showed early fibrosis and thickening. Later reports (6, 7) discussed further observations of similar experiments in cats, with a description of the pathological changes in the liver and speculations about the origin of the ascitic fluid.

In 1930 Zimmerman and Hillsman (18) reported a series of similar vena caval obstructions in dogs, using an aluminum band as the constricting agent. They found that necrosis of the liver followed the simple mechanical obstruction, the extent of the necrosis being proportional to its amount. Repair was found to begin as early as 6 days after partial ligation and to progress to fibrosis about the central veins, with marked distention of the subcapsular venous sinusoids. Fibrosis increased for about 85 days following ligation and remained stationary at 216 days, which was the duration of the longest experiment. The conclusion from these experiments was that central fibrosis could result without infection and was due to active proliferation of connective tissue.

Simonds and Callaway (16) reported anatomical changes in the livers of dogs, following mechanical constriction of the hepatic veins, and Armstrong and Richards (1) tied the hepatic veins in the abdomen and their dogs developed an anemia and hypoproteinemia.

Kershner *et al.* (11) described a three-stage procedure for ligating the inferior vena cava above and below the liver for the purpose of studying the anatomy of hepatic veins. They described the anatomic relationships between the hepatic veins and the posterior vena cava in the dog. The method apparently carried a high mortality with only four of forty-six dogs used surviving the series of operations.

Methods

The dogs used in these experiments were healthy animals which had been maintained on a kennel diet of hospital scraps for many months prior to the studies. They were placed in metabolism cages in a room convenient for constant observation. Dog 42-893, a beagle mongrel female, was used in a pilot experiment to determine the effects of the operation and postoperative course. Consequently limited baseline studies were carried out as far as

the nitrogen balance was concerned, emphasis being placed on the blood protein determinations, weight, and fluid intake and output. As the experiment progressed and the dog's health remained essentially unchanged, additional determinations were added to include urinary nitrogen determination, fibrinogen levels, occasional fecal nitrogen studies, and later sodium chloride studies. The second dog, 40-37, a mongrel spaniel male, obstructed at a later date, presents a more adequate preparatory study including nitrogen balance studies.

The operation performed to constrict the vena cava is comparatively simple owing to the anatomic relationship of the dog in which a 3 inch segment of vena cava is present between the diaphragm and the right auricle. The animal was anesthetized by nembutal intravenously (1 grain per 2.4 kg. body weight). The right chest was entered through an incision between the 6th and 7th ribs, proper hemostasis and asepsis being observed. Oxygen under positive alternating pressure (8) was used to maintain inflation of the right lung during the intrathoracic portion of the procedure. A self-retaining retractor provided an ample field for application of the constricting band, thereby avoiding a rib resection procedure. The vena cava was freed from its membranous attachments, the phrenic nerve dissected away, and calipers inserted to determine the diameter of the vessel. The constricting band, consisting of a strip of thin pliable aluminum 1 cm. in width, was then applied and the vessel narrowed to about one-half to one-third its former diameter, that is, from 1.0–1.2 cm. to 0.4–0.5 cm. The wound was then closed in layers, a jacket applied to the thorax, and the oxygen discontinued. Ascitic fluid accumulation began rather promptly in all cases.

Determinations of plasma protein and ascitic fluid protein were made by macro-Kjeldahl method. Plasma albumin and ascitic fluid albumin were measured by the methanol precipitation method of Pillemer and Hutchinson (15), followed by macro-Kjeldahl study of the filtrate. Fibrinogen was determined by the micro-Kjeldahl analysis of the clot following calcium chloride precipitation. Urinary nitrogen was determined on 48 hour samples by the macro-Kjeldahl method. Fecal nitrogen determinations were made on 1 week pooled specimens, but were discontinued after several determinations yielded weekly amounts of less than 3 gm. total nitrogen.

The *low protein diet* consisted of 100 gm. of a sucrose, lard, bone ash mixture, with added vitamins plus 30 gm. of cooked horsemeat and had a daily nitrogen content of 1.4 gm. Ferric citrate (600 mg. iron) was added daily.

The *high protein diet* consisted of 300 gm. of cooked horsemeat with added vitamins and ferric citrate and contained 13.4 gm. of nitrogen daily. Soy bean meal was used in one case (Table 1). This diet consisted of 200 gm. of cooked soy bean meal mixed with 200 gm. of cooked horsemeat and having a daily nitrogen content of 13.6 gm.

Nitrogen balance was determined by subtracting nitrogen output (ascitic fluid and urine) from nitrogen intake (diet).

Electrophoretic studies of plasma and ascitic fluid were performed by Dr. Eric Alling.

EXPERIMENTAL OBSERVATIONS

The two dogs followed basically the same postoperative course. Recovery from operation was uneventful. A few days after operation, the presence of intraperitoneal fluid was detected and weekly paracenteses were instituted. In one dog on which the operation was first tried to explore difficulties in technique, about 1 liter of clear yellow-tinged fluid was found in the peritoneal cavity when the animal was killed under ether anesthesia 5 days following application of the band. During the recovery period, the dogs were maintained on the usual kennel diet of hospital scraps. The ascitic fluid removed was reddish with a hematocrit of about 1.0 to 0.2 per cent. It was rich in protein and formed a jelly-like clot on standing. Electrophoretic studies showed the ascitic fluid to be of identical pattern with the plasma protein, though at lower levels, with elimination of the fibrinogen peak in the ascitic fluid (as a result of the clot formation).

Collateral circulation became evident in 4 to 6 weeks following operation. This was more evident in the female of the two dogs studied, since this dog had well developed mammary vessels, presumably because she had had pups. During the postoperative period it was noted that the plasma protein levels diminished gradually and that the ascitic fluid gradually

TABLE 1

Contrasting Effects of High Protein and Kennel Diets (Poor Protein)
(Dog 42-893)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed	
				Plasma proteins		Ascitic fluid proteins		Volume	Total protein
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.
1	High protein	10.0	32.7	6.9	1.8	—	—	—	—
		9.7	34.0	7.4	1.4	5.7	1.1	520	29.6
2	High protein	10.1	30.9	8.1	1.1	—	—	—	—
		10.1	34.5	7.1	1.2	6.1	1.0	400	24.0
3	High protein	10.6	37.0	7.5	1.2	—	—	—	—
		9.7	35.3	6.7	1.2	6.0	1.3	1450	87.0
4	High protein	9.2	34.9	7.4	1.4	—	—	—	—
		9.6	36.1	7.5	1.1	6.3	1.0	400	25.2
5	Kennel (poor protein)	11.0	36.2	5.4	1.0	—	—	—	—
		10.5	35.0	4.8	1.5	4.3	1.3	2500	107.5
6	Kennel (poor protein)	12.7	—	5.4	1.0	3.6	0.8	1850	66.6
		12.3	35.3	5.2	1.0	—	—	—	—
7	Kennel (poor protein)	11.6	37.0	4.9	1.1	3.4	1.0	2000	68.0
		10.9	34.0	4.8	1.1	3.5	1.0	2540	88.9
8	Kennel (poor protein)	11.8	29.8	4.9	0.9	—	—	—	—
		11.0	30.2	5.5	1.1	3.4	1.2	1820	61.9
9	Kennel (poor protein)	11.9	33.4	5.6	1.3	—	—	—	—
		11.4	30.5	4.8	1.1	3.5	1.2	2350	82.2

increased. At that time the kennel diet was largely a mixture of vegetable material and grains (wheat and rice), and though not analyzed was definitely on the low protein side. About 5 weeks after the operation the dog, 42-893, was placed on a high protein diet consisting of 200 gm. cooked soy bean meal, 200 gm. of cooked horsemeat, added vitamins, and ferric citrate solution, with a daily nitrogen intake of 13.6 gm.

Table 1 presents a contrast between the high protein diet and the kennel diet in the maintenance of plasma protein concentration and the formation of

ascitic fluid in dog 42-893. On the high protein diet, the plasma protein concentration was maintained at about 7.0 gm. per cent except on a single occasion, while the protein concentration of the ascitic fluid ranged in the neighborhood of 6.0 gm. per cent with comparable A/G ratios. The volume of ascitic fluid was fairly low except for 1 week (period 3) when 1450 cc. were withdrawn. This discrepancy may be explainable on the basis of later findings regarding the importance of sodium chloride intake, since the dog received an undialyzed hambone in addition to its usual rations during period 3. Protein in amounts of 29.6, 24.0, 87.0, and 25.2 gm. respectively, an average of 41 gm. per week, was removed in the ascitic fluid. Hematocrit changes were negligible.

After 4 weeks on the high protein diet, the animal was returned to the kennel diet. The effect of this diet on the plasma protein concentration was remarkable, the level ranging between 4.5 and 5.5 gm. per cent during the 5 weeks of the experiment. During this period the amount of ascitic fluid increased strikingly, necessitating semiweekly paracenteses in some instances. The ascitic fluid protein level fell to and was maintained below 3.5 gm. per cent, and protein in amounts of 107.5, 66.6, 156.9, 61.9, and 82.2 gm. respectively, an average of 95 gm. per week, was removed during the last 5 weeks. Hematocrit readings showed development of slight anemia, and weight increase was commensurate with accumulation of intraperitoneal fluid.

Observation of the increased amount of ascitic fluid produced while the dog was maintained on the kennel diet, indicated the necessity for nitrogen balance studies. Consequently (Table 2), the dog was shifted to the high protein diet of 300 gm. of cooked horsemeat daily. The animal was next given a diet of sucrose, lard, bone ash, and vitamin supplements with 30 gm. of horsemeat and 600 mg. of iron as ferric citrate. The nitrogen content was 1.4 gm. daily.

Table 2 presents a continuation of Table 1. The high plasma protein concentration and low ascitic fluid output were again prominent results of the high protein diet. Nitrogen balance was strongly positive. After 3 weeks on this diet without remarkable change in hematocrit or fibrinogen readings, the low protein diet was started. The pattern was somewhat different from that when the dog was on the kennel diet. The plasma protein level fell slowly and after 2 weeks was still above 6 gm. per cent. Ascitic fluid production was not conspicuous with protein concentration of 4.9 and 4.4 gm. per cent respectively for the 2 weeks and total protein removed of 31.9 and 36.9 gm. for the two periods. These amounts were about one-third of those recorded when the animal was on the kennel diet. It was assumed therefore that factors other than protein intake were in part responsible for the variation in fluid accumulation. One of these factors was presumed to be sodium chloride. Consequently the animal was continued on the low protein diet and in addition was given 6 gm. of sodium chloride in capsule form daily (periods 6 and 7). Immediately

the ascitic fluid accumulation increased, fluid relatively poor in protein, while the blood plasma protein concentration dropped below 4.5 gm. per cent. During the 2 week period while the animal was maintained on low protein diet with added sodium chloride, 5390 cc. of ascitic fluid containing 110.5 gm. of protein, and average of 55.2 gm. weekly, were removed. In period 7 the dog refused

TABLE 2

Contrasting Effects of High Protein Diet, Low Protein Diet, and Sodium Chloride Added to Low Protein Diet

(Dog 42-893)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed		Fibrinogen	Nitrogen balance gm.		
				Plasma proteins		Ascitic fluid proteins					Intake	Output	
								Volume	Total protein		Diet	Ascites	Urine
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.	mg. per cent			
1	High protein	9.4	33.6	7.1	2.0	—	—	—	—	366	93.6	6.5	40.6
		9.6	32.0	6.8	1.3	4.9	0.9	810	39.9	—			
2	High protein	9.9	32.5	8.0	1.1	—	—	—	—	358	93.6	—	43.3
		9.7	30.0	7.8	1.0	—	—	—	—	360			
3	High protein	10.2	34.2	8.2	1.1	—	—	—	—	300	93.6	2.5	65.7
		10.0	34.8	7.7	1.1	5.4	1.2	300	16.2	332			
4	Low protein	10.5	37.9	7.0	1.4	—	—	—	—	302	8.2	5.0	14.8
		10.0	37.0	6.6	—	4.9	1.9	650	31.9	245			
5	Low protein	10.0	39.8	6.5	1.4	—	—	—	—	—	6.6	6.0	18.5
		9.3	37.4	6.2	1.2	4.4	1.3	840	36.9	208			
6	Low protein	10.5	27.0	4.9	1.6	—	—	—	—	212	4.9	9.1	12.3
	plus NaCl	10.2	31.5	4.3	2.2	2.6	3.3	2020	56.8	280			
7	Low protein	10.7	33.0	4.1	1.1	1.9	20.0	2220	42.2	—	0	8.5	10.8
	plus NaCl	11.0	32.0	4.3	—	1.0	9.0	1150	11.5	310			

all of the low protein diet but the sodium chloride was continued. This state of affairs provided a contrast to that of the previous 2 weeks when the animal was receiving the low protein diet alone and produced 1490 cc. of ascitic fluid containing 68.8 gm. of protein, an average of 34.4 gm. weekly. Changes in the hematocrit findings were negligible and fibrinogen levels trended downwards as the plasma protein concentration fell. Note the very high A/G ratio due to great albumin preponderance in period 7.

Table 3 illustrates a similar series of experiments performed on the second dog, 40-37. During the 2 week period while the animal was on the high protein diet the plasma protein level was maintained above 7.0 gm. per cent, no ascitic fluid was produced, and a positive nitrogen balance was recorded.

TABLE 3

Contrasting Effects of High Protein Diet, Low Protein Diet, and Sodium Chloride Added to Low Protein Diet

(Dog 40-37)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed		Fibrinogen	Nitrogen balance gm.			Intake minus output
				Plasma proteins		Ascitic fluid proteins					Intake	Output		
								Volume	Total protein		Diet	Ascites	Urine	
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.	mg. per cent				
1	High protein	10.2	41.7	7.0	1.0					244	62.4	—	42.6	19.8
		10.0	37.1	7.2	1.4					293				
2	High protein	10.0	35.4	7.4	1.0					268	53.5	—	39.6	13.9
		10.0	33.2	7.0	1.0					305				
3	Low protein	10.0	31.2	6.3	1.2					545	18.4	—	29.5	−11.1
		10.5	32.1	6.2	0.7					496				
4	Low protein	10.7	31.5	5.4	0.9					311	11.1	—	8.3	2.8
		10.5	36.1	4.7	1.0					—				
5	Low protein	10.7	44.6	5.3	0.6					325	11.1	—	13.9	−2.8
		11.1	44.9	5.7	0.6					364				
6	Low protein plus NaCl	11.6	45.8	3.6	1.7	2.8	0.9	2000	56.0	283	11.1	15.8	22.9	−27.4
		11.6	47.0	3.5	1.4	1.8	1.7	2500	45.0	239				
7	Low protein plus NaCl	10.0	50.9	4.1	1.0	1.6	1.0	1830	29.3	315	4.7	4.5	9.4	−9.2

During the following 3 weeks the dog ate a low protein diet containing 1.58 gm. of nitrogen daily. The animal's weight increased 1.1 kg. and the plasma protein level fell gradually so that at the end of the period it was 5.7 gm. per cent. This weight increase in large part surely represented the accumulation of ascites, and some of the 2000 cc. removed in the first part of period 6 was related to this reaction. The overall nitrogen balance for the low protein period was negative with output exceeding intake by 11.1 gm.

During the following 10 days (periods 6 and 7) the animal received 6 gm. of

sodium chloride daily in addition to the low protein diet. The plasma protein concentration fell below 4.0 gm. per cent, and was 4.1 gm. per cent at the final determination. During this 10 day period 6330 cc. of ascitic fluid containing 130.3 gm. of protein were removed, an average of 86.8 gm. weekly. Nitrogen for the period showed a 36.6 gm. negative balance. Hematocrit showed a rise during the latter part of the low protein period, and during the time when the sodium chloride was added to the diet. This might well be a hemoconcentration associated with a drain of plasma protein and reduction of the circulating plasma volume. Fibrinogen figures showed a downward trend during the period of lowered plasma protein concentration. The results of this series of experiments agreed with those presented in Tables 1 and 2.

In view of the remarkable change in both circulating plasma protein level and ascitic fluid production brought about by the liberal addition of sodium chloride to the diet, it seemed pertinent to try a sodium-free salt mixture as another means of checking the relationship of the sodium ion to ascitic fluid production. Through the courtesy of the Winthrop Chemical Company, we were able to obtain a supply of such a sodium-free mixture.[1]

After a 3 week rest period on a high protein diet, the final week of which is recorded in Table 4, dog 42-893 was placed on the standard low protein diet for 2½ weeks. The results of these two trials agreed with previous data. The plasma protein level stabilized at slightly under 6 gm. per cent and the nitrogen balance was slightly negative. After this introductory program, 6 gm. of sodium-free salt mixture in capsule form were given in addition to the low protein diet for a 10 day period. The plasma protein level did not change perceptibly during this period, remaining slightly below 6 gm. per cent. The animal produced 670 cc. of ascitic fluid containing 19.7 gm. of protein, an average of 13.1 gm. weekly, and was in slightly negative nitrogen balance for the period. There were no toxic effects from the neocurtesal, and animal remained alert, active, and in good health. No significant changes in weight, hematocrit, or fibrinogen level were noted.

Immediately following this 10 day period, the dog was continued for a 7 day period on the low protein diet, but with 6 gm. of sodium chloride replacing the 6 gm. of sodium-free salt mixture. Immediately the plasma protein level began to fall, and continued to fall steadily reaching a level of 4.2 gm. per cent at the end of the period. Increased amounts of ascitic fluid were formed and 4250 cc. containing 61.0 gm. of protein were removed. The nitrogen balance was more definitely negative, while the weight increased due to the fluid accumulation, and the hematocrit readings remained at the same anemic level.

A follow-up week of high protein diet was also recorded in this table to

[1] The trade name for the mixture is "neocurtesal." The formula for the compound is potassium chloride 66.0 per cent, ammonium chloride 12.0 per cent, starch 17.0 per cent, potassium formate 3.0 per cent, calcium formate 1.0 per cent, and magnesium citrate 1.0 per cent. The compound has a saline taste.

illustrate the prompt clearing of ascites, and the recovery of plasma protein to former normal concentrations, with the attending positive nitrogen balance.

After a 5 week period of rest on a high protein diet, the last two weeks of

TABLE 4

Contrasting Effects of Sodium-Free Salt Mixture and Sodium Chloride Added to Low Protein Diet

(Dog 42-893)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed		Fibrinogen	Nitrogen balance gm.			Intake *minus* output
				Plasma proteins		Ascitic fluid proteins					Intake	Output		
								Volume	Total protein		Diet	Ascites	Urine	
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.	mg. per cent				
1	High protein	10.0	28.8	7.4	0.7	—	—	—	—	—	93.6	2.2	57.1	34.3
		10.2	30.5	7.0	0.9	3.8	0.6	360	13.7	286				
2	Low protein	10.0	29.7	6.6	0.7	—	—	—	—	—	11.1	—	14.5	−3.4
		10.0	27.7	6.3	0.7	—	—	—	—	—				
3	Low protein	10.0	28.7	6.1	0.7	3.3	1.5	675	22.3	265	11.1	5.8	12.6	−7.3
		9.8	31.2	5.9	0.4	3.0	0.9	440	13.2	254				
4	Low protein	10.0	27.3	5.9	0.7	—	—	—	—	295	6.3	—	6.4	−0.1
	Low protein plus sodium-free salt	9.6	29.6	5.7	0.6	2.9	1.3	410	11.9	234	6.3	1.9	6.1	−1.7
5	Low protein plus sodium-free salt	9.6	30.5	5.9	0.4	—	—	—	—	274	11.1	1.2	14.6	−4.7
		9.2	29.7	5.9	0.6	3.0	0.7	260	7.8	—				
6	Low protein plus NaCl	11.5	31.7	5.0	0.5	1.9	1.0	950	18.1	332	11.1	9.5	13.6	−12.0
		10.9	31.0	4.2	0.8	1.3	0.5	3300	42.9	—				
7	High protein	10.5	29.3	6.1	0.8	—	—	—	—	—	93.6	—	27.7	65.9
		10.0	28.8	7.0	0.7	—	—	—	—	—				

which are shown in Table 5, the dog was given 6 gm. of neocurtesal daily in addition to the high protein diet for a week period. The plasma protein level did not change from its former range of over 7.0 gm. per cent, there was no ascitic fluid production, and the positive nitrogen balance increased.

During the next 2½ weeks (periods 4 to 6, Table 5) 6 gm. of sodium chloride daily was added to the high protein diet. A definite change in all the deter-

minations occurred almost immediately. The plasma protein concentration fell from 7.3 gm. per cent to 4.9 gm. per cent in spite of nitrogen intake of over 13 gm. per day. The ascitic fluid production was active and 5235 cc. containing

TABLE 5

Contrasting Effects of Sodium-Free Salt Mixture and Sodium Chloride Added to High Protein Diet

(Dog 42-893)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed		Fibrinogen	Nitrogen balance gm.			Intake minus output
				Plasma proteins		Ascitic fluid proteins		Volume	Total protein		Intake	Output		
											Diet	Ascites	Urine	
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.	mg. per cent				
1*	High protein	10.5	32.3	7.2	0.6	—	—	—	—	380	71.5	—	64.3	7.2
2	High protein	10.7	35.1	6.8	0.5					—	53.5	—	51.2	2.3
		11.0	34.8	7.3	0.4					—				
3	High protein	11.1	35.3	7.1	0.7					—	91.4	—	64.3	27.1
	plus sodium-free salt	10.5	35.1	7.3	0.4					283				
4	High protein	11.6	32.2	6.5	0.9	3.9	1.7	470	18.3	273	87.9	9.8	62.7	15.4
	plus NaCl	10.9	35.1	5.9	0.8	3.4	—	1250	42.5	—				
5	High protein	11.1	38.4	5.7	—	3.1	—	1060	32.8	275	93.6	12.3	66.1	15.2
	plus NaCl	10.5	37.1	5.6	0.9	2.8	—	1575	44.1	237				
6	High protein plus NaCl	10.7	—	4.9	1.1	2.7	—	880	23.8	285	40.1	3.8	25.7	10.6
	High protein	10.6	38.8	5.4	0.4					268	53.5	—	30.6	22.9
7	High protein	10.7	35.5	6.3	0.8					302	60.2	—	53.4	6.8
		10.9	32.1	6.0	0.6					237				

* 3 weeks of high protein diet preceding.

161.5 gm. of protein, a weekly average of 64.6 gm., were removed. The nitrogen balance remained positive. Hematocrit and fibrinogen values did not change.

A 10 day recovery period on a high protein diet alone showed a gradual return toward a normal plasma protein concentration with an immediate drying up of the ascitic fluid. The urinary nitrogen output returned to the levels of periods 1 and 2.

TABLE 6

Contrasting Effects of Sodium-Free Salt Mixture and Sodium Chloride Added to High Protein Diet

(Dog 40-37)

Period 7 days	Diet	Weight	Hematocrit value	Concentration				Ascitic fluid removed		Fibrinogen	Nitrogen balance gm.			Intake minus output
				Plasma proteins		Ascitic fluid proteins					Intake	Output		
								Volume	Total protein		Diet	Ascites	Urine	
		kg.	per cent	gm. per cent	A/G	gm. per cent	A/G	cc.	gm.	mg. per cent				
1	High protein	9.1	38.6	8.6	1.4	—	—	—	—	377	65.1	—	28.0	37.1
		8.8	37.2	8.3	1.3	—	—	—	—	342				
2	High protein	8.8	37.5	8.1	2.0	—	—	—	—	296	51.3	—	29.5	21.8
		8.8	30.5	7.9	—	—	—	—	—	336				
3	High protein plus sodium-free salt	8.9	30.2	7.7	1.1	—	—	—	—	340	58.0	—	30.4	27.6
		8.9	32.6	7.4	1.1	—	—	—	—	333				
4	High protein plus sodium-free salt	8.9	34.0	6.9	1.4	—	—	—	—	324	31.2	—	10.8	20.4
	High protein	9.6	35.4	7.0	1.2	—	—	—	—	354	98.0	—	44.5	53.5
	(6 wks. on high protein diet not recorded)													
5	High protein	9.6	33.8	8.0	0.7	—	—	—	—	266	40.1	—	19.3	20.8
6	High protein	10.0	32.8	8.6	0.6	—	—	—	—	326	84.7	—	38.7	46.0
	High protein	10.2	34.0	8.3	1.2	—	—	—	—	461				
7	High protein	10.7	35.5	8.0	0.6	—	—	—	—	322	51.3	—	29.9	21.4
	High protein plus NaCl	10.9	35.9	5.6	1.7	4.7	—	700	32.9	—	40.1	5.3	30.2	4.6
8	High protein plus NaCl	11.2	36.8	6.2	1.3	4.2	—	220	9.2	—	53.5	1.5	43.3	8.7
	High protein plus NaCl	11.3	40.3	5.5	1.0	3.7	—	390	14.5	280	40.1	2.3	27.4	10.4
9	High protein plus NaCl	11.1	38.8	6.1	1.0	3.3	—	180	5.9	354	53.5	0.9	35.3	17.3
	High protein	10.9	39.9	6.9	0.9	—	—	—	—	378	40.1	—	17.3	22.8

Table 6 illustrates a similar group of experiments on dog 40-37. The addition of 6 gm. daily of neocurtesal to the high protein diet caused insignificant

differences in the plasma protein level for the 10 day period with no detectable fluid production and no great change in the nitrogen balance.

After an interval of eight weeks on a high protein diet alone, the final two of which are shown in periods 5, 6, and 7 in the table, the dog was given 6 gm. of sodium chloride in addition to the horsemeat diet. The amount of ascitic fluid produced was not as large as noted in the first dog, probably because of the increasing development of collateral circulation and lessened portal stasis, but nevertheless significant changes were present. The plasma protein concentration dropped from 8 gm. per cent down to below 6.0 gm. per cent in the 2 week period with the production of 1490 cc. of ascitic fluid containing 62.5 gm. of protein. The nitrogen balance remained positive. This fluid production took place after 3 months of complete freedom from ascites.

In addition to the experiments recorded above, a few other pertinent observations are available.

Dog 40-37, which had the constricting band placed about its inferior vena cava on December 18, 1946, was reoperated upon January 7, 1948, at which time the vena cava was ligated completely and sectioned. The dog remained well for 9 days following this procedure, and then became increasingly inactive, refused food, and died 13 days after the vein section. At autopsy, about 1500 cc. of ascitic fluid was found. The collateral vessels were well developed in the omentum, with adhesions to the peritoneal surfaces, in the mediastinum, and throughout the pleural surfaces of the diaphragm. Azygos veins were greatly enlarged. Numerous adhesions, some of them of recent fibrinous nature, were present about the liver. The cause of death was ascertained to be acute pyloric ulcers with massive intestinal hemorrhage.

The liver weighed 500 gm. The capsule showed numerous areas of greyish fibrosis and there were interlobar adhesions and adhesions to the diaphragm and surrounding structures of both fibrinous and fibrous nature. The parenchyma showed numerous large nodular deformities. On section the organ showed a subcapsular zone measuring about 0.5 cm. in width which was markedly congested. There were numerous nodular areas of paler regenerating tissue intermingled with dark red congested tissue. In general the central areas appeared dark red and dilated against a yellowish grey background.

Histologically, the lobular structure was fairly well preserved. The subcapsular sinusoids were greatly dilated so that the picture was almost hemangiomatous in some places. The subcapsular liver cells were large and pale and contained considerable glycogen. The remainder of the parenchyma showed numerous focal necroses and the portal vessels were greatly dilated in every case.

Another experimental animal, dog 44-180, on which a similar procedure to constrict the vena cava was performed on April 17, 1947, and a second procedure with complete ligation and section of the vena cava on July 18, 1947, was sacrificed on November 22, 1947, a period of 7 months intervening since the initial operation. At autopsy there was no ascites. The collateral vessels were well developed in the mediastinum, in the omentum, and in adhesions about the liver. The azygos and intercostal veins were greatly dilated. The viscera in general were within normal limits.

The liver weighed 660 gm. The external surface showed a few macular areas of greyish fibrosis measuring up to 2 cm. in diameter. Numerous web-like adhesions were present be-

tween the various lobes. The organ was dark colored and congested. It was quite firm and cut with resistance. The cut surface showed a regular pattern with small greyish white areas against a reddish purple background. In some areas there was a yellow mottling indicating a fatty change. Intrahepatic vessels were dilated. In some areas the parenchyma was slightly elevated above the usual surface, and in these areas there was regenerative change. The gall-bladder was smooth and pale and contained about 10 cc. of clear bile. The extra hepatic ducts were patent.

Histologically, the liver was in general close to normal. The liver cells contained much glycogen and scattered fat droplets. No necrosis was observed. Glisson's capsule, bile ducts, and portal structures were normal. There was a marked general and central congestion. The stroma of the central portion of the liver lobules was somewhat thickened, but no large scars and no irregularity of lobulation were observed.

These livers thus resembled those described by Bolton (4), and Zimmerman and Hillsman (18)—a severe passive congestion with perhaps some early central fibrosis.

Liver function tests showed negative thymol turbidity tests after more than 1 year in dog 42-893 and dog 40-37. Bromsulfalein clearances showed a 6 per cent retention in dog 42-893 after 9 months, and 7 per cent retention in dog 40-37 after 6 months. Dog 44-180 showed a 2 per cent bromsulfalein retention and a negative thymol turbidity 3 days before sacrifice. Blood chloride determinations fell within normal limits, and when the animal was on a diet with extra sodium chloride added, the ascitic fluid chlorides rose as the ascitic fluid proteins declined.

DISCUSSION

Is there a circulation of ascitic fluid—in other words is the ascitic fluid being constantly added to from the portal circulation and constantly absorbed by the lymphatic apparatus in the diaphragm and pelvis? It seems almost certain that some absorption of ascitic fluid takes place since the normal peritoneal cavity promptly removes introduced fluid, plasma, and even red cells (9) in considerable amounts. In a dog with 1000 cc. of ascitic fluid one would wish to know just how rapid this absorption might be. It is not safe to assume that when ascites is accumulating the excess fluid means the algebraic sum of the ascites extravasated less the total absorption from a *normal* peritoneal cavity. This question can be approached experimentally and some work is in progress.

Plasmapheresis in standard hypoproteinemic dogs (10) shows that 70 gm. new plasma production per week is a high output for a 10 kg. dog. It is obvious from many of the experiments tabulated above (Tables 1 and 3) that this experimental ascites and the resulting *internal plasmapheresis* may produce 95 gm. of new plasma protein in the ascitic fluid removed each week (average of the last 5 weeks (Table 1) on kennel diet). This large protein output may be in part a carry over from the preceding high protein diet periods, but, allowing for this and the drop in the concentration of the circulating plasma proteins, we have at least a normal high plasma protein output by way of the

removed ascitic fluid. This of course suggests that the portal stasis has not seriously embarrassed the function of the hepatic epithelium which is important in the plasma protein production.

The influence of the sodium ion upon the accumulation of ascitic fluid, as observed in cardiac dropsy, is obvious in these experiments. We were surprised by the amount of the resulting ascitic fluid. The amount of protein in the diet is likewise a factor, and a high protein intake may eliminate an ascites which persists on a moderate or low protein intake. The changes in the circulating plasma protein concentration are definite and result from the loss of plasma protein by way of the ascites and the lessened production of new plasma protein from the low protein diet. There is room for study of palatable sodium-free seasoning agents which have no injurious after-effects. In simple portal stasis in humans, it may be possible to control the accumulation of ascites by meticulous attention to the sodium intake and the protein content of the diet.

SUMMARY AND CONCLUSIONS

Constriction of inferior vena cava above the diaphragm is used to produce experimental ascites in the dog.

This type of experimental ascites drains the body protein reserves, reduces the level of circulating plasma proteins, and in effect is an *internal plasmapheresis.*

As the ascitic fluid is withdrawn and the proteins measured, we observe a production of ascitic protein (80–90 gm. per week) comparable to that removed by plasmapheresis (bleeding and replacement of red cells in saline).

High protein diet tends to decrease the ascites but the protein content of the ascitic fluid may increase.

Sodium chloride increases notably the volume of the ascites which accumulates and the total ascitic protein output increases. Sodium-free salt mixtures have a negative influence.

High protein diet low in sodium salts gives minimal ascitic accumulation under these conditions.

The question of circulation of the ascitic fluid is raised—how rapid is the absorption and the related accumulation?

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THE DETERMINATION OF THE CONCENTRATION OF THE DYE T-1824 IN NORMAL AND LIPEMIC PLASMAS

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The customary method of reading the optical density of a dye-stained plasma sample at a standard wave length against dye-free plasma at the same wave length is unsatisfactory in very lipemic plasma for three main reasons: (1) Few spectrophotometers are available which can accurately determine relatively small differences in optical density at high optical densities, such as are encountered in turbid, lipemic plasmas. (2) There appears to be a specific effect of the suspended fat particles in their scattering of light. This scattering results in changes in the T-1824 extinction-concentration curves which are thereby different in lipemic and non-lipemic plasmas. The difference increases with the degree of lipemia though the increase is not linear. (3) At high lipid particle concentrations the fat particles appear to be unstable and tend to coalesce and rise; optical densities of unstained samples of the same lipemic plasma may vary by as much as 10 per cent, so that the blank optical density cannot be measured precisely. Calculations of plasma volumes by the dye method with lipemic plasmas have been reported to give results which are completely erroneous (1), particularly when there is a difference in the degree of lipemia between blank and dye-stained plasmas. Such changes in the degree of lipemia are particularly marked following the intravenous administration of human albumin (2).

Several procedures have been suggested to surmount this difficulty. Luetscher (2) has recommended measuring the optical densities of plasmas at the usual wave length (λ = 620 mμ) and another "indifferent" wave length, (*e.g.* λ = 540 mμ) at which the dye absorbs relatively little light, to compensate for changes in lipemia. This, however, has been found to be impractical with plasmas where the blank optical density is 1.5 or higher in the cuvettes used (cylindrical, about 10 mm. internal diameter) at λ = 620 mμ because the blank optical density increases markedly with decrease in wave length. For photometric measurement Harington, Pochin, and Squire (3) have suggested that the dye be extracted from plasma into butyl alcohol by a procedure which is complicated and is affected by hemolysis. Crooke and Morris (4) have used the filtrate from precipitation of the plasma proteins and lipids with a hydrochloric acid-ethanol-phosphotungstic acid reagent; a correction is required in the presence of hemolysis. In addition the supernatants are not always clear and fading of the dye occasionally occurs, because of an undetermined impurity

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in the phosphotungstic acid (5). Morris (5) has used chromatographic adsorption of the dye from plasma adjusted to pH 10 onto a column of alumina, followed by elution with acetic acid and ethanol. This procedure, too, is affected by hemolysis and requires an elaborate apparatus. Another method based on the reduction of T-1824 by $Na_2S_2O_4$ in alkaline solution has been reported from this laboratory by Phillips (6). The results obtained by this method are not affected by hemolysis and are apparently quite reliable. A type of spectrophotometer more precise than may be generally available is required and it is necessary to wait 12 hours to finish a determination. Further, Phillips' method cannot correct (*a*) for the change in optical density which may occur as a result of the rise of the fat particles to the top of the plasmas after the plasmas have been exposed to the cold for a few hours, (*b*) for the possible coalescence of the fat particles, and (*c*) for the effect of the lipemia in spite of the 1:5 dilution. Other procedures, such as fat extraction with ethylene-dichloride and centrifugation at 18,000 R.P.M., have been reviewed by Gregersen (7) but were found unsatisfactory in the presence of marked lipemia. Gibson, quoted by Thorn (1), has unsuccessfully tried extraction of the fats with triethyl phosphate.

The present paper reports a procedure which permits the determination of T-1824 in lipemic plasmas. It is based on addition of acetone, which dissolves the lipids,[1] extracts[2] the T-1824, and precipitates the plasma proteins. A small fraction of the dye, varying with the plasma protein concentration, remains bound in the precipitated protein. Because of this, it is necessary to prepare a reference solution (8) of the dye in the subject's plasma for each determination. The method is relatively little affected by hemolysis, and, since the lipid particles disappear, is not at all affected by changes in the degree of lipemia. Approximately 1 hour is required for a complete determination.

The method was developed to study plasma and blood volume changes in nephrotic children treated with intravenous human albumin; it is not proposed as a substitute for the usual procedures in the absence of lipemia. Its reliability has been checked in normal individuals and with normal plasmas but not in diseases other than the nephrotic syndrome. The general procedure and precautions for the determination of plasma volume and whole blood volumes with T-1824 have been set forth in detail in the publications of Gregersen and his coworkers and those of Gibson and his associates.

Materials and Technic

Reagents

Acetone, analytical reagent.

T-1824, 0.5 per cent solution in glass ampoules.[3]

NaCl, U. S. P. grade, 0.85 per cent in distilled water.

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[1] Determination of total lipid carbon in plasma and the acetone extract shows that approximately 50 per cent of the total lipid is extracted in the acetone.

[2] "Extracts" is used advisedly. In plasma, the T-1824 is bound to the albumin fraction and is removed from the albumin by the acetone which precipitates the protein. Most of the T-1824 remains in solution in the acetone.

[3] Procurable from the William R. Warner Co., New York.

Procedure[4]

Drawing and Handling of Blood.—Approximately 4 ml. of blood is drawn and transferred to a test tube containing dried heparin. Heparin is preferred as anticoagulant; if oxalate is used the amount should not exceed 1 or at most 2 mg. per ml. of blood. Larger amounts of oxalate may cause hemolysis. The needle is left in place in the vein and a Krogh-Keys syringe pipette,[5] containing the dye, is adapted to the needle; the dye is injected at one stroke. The Krogh-Keys syringe pipette is not washed with blood after the delivery. The syringe and needle are then removed together. After the required time interval (in this laboratory both 10 minute intervals and the falling curve method have been used) another sample of blood[6] is drawn.

The blood samples, after aliquots have been set aside for hematocrit determinations by centrifugation, are centrifuged at 3000 R.P.M. for 10 minutes and the plasmas separated with care to avoid drawing up red blood cells. Very lipemic plasmas may require longer or repeated centrifugation.

One ml. of the dye-stained plasma and two samples of the unstained plasma are pipetted accurately into 12 × 75 mm. Pyrex tubes. To each of the dye-stained samples and to one of the dye-free samples is added, from a 2 ml. burette, exactly 0.2 ml. of 0.85 per cent NaCl.

Preparation of Standard.—The syringe previously used for the intravenous injection of the dye is now refilled with dye solution to the same mark used for the injection and is fitted to a needle filled with saline solution. The dye solution in the syringe is then injected into a volumetric flask suitable to permit exact dilution with 0.85 per cent NaCl to a final volume roughly one-fifth the expected plasma volume. For example, if the expected plasma volume is 2500 ml., a 500 ml. volumetric flask is used. To have the same dead space in the needle it is necessary to use the same needle that was employed for the intravenous injection.

The standard solution is prepared by adding of the diluted dye solution exactly 0.2 ml. to 1 ml. of one of the unstained plasmas in a 12 × 75 mm. Pyrex tube.

Measurement of Dye Concentration in the Plasma

There are now 3 tubes prepared, which are designated as follows: a, the plasma blank, *viz.* plasma without dye; D_B, the standard prepared by adding dye to plasma *in vitro*; D_V, the dye-stained plasma drawn from the subject 10 minutes after the intravenous dye injection.

The contents of all 3 tubes are gently swirled to ensure mixing, and to each is added 3.0 ml. of acetone from a 25 ml. burette. The stopcock of the burette is greased with a silicone lubricant. As each tube receives the acetone it is closed with a rubber stopper and shaken vigorously for 10 seconds. The tubes, still stoppered to prevent evaporation of acetone, are

[4] The procedure may be applied when necessary to 0.5 ml. samples of plasma. In that case only 0.1 ml. of 0.85 per cent NaCl, 0.1 ml. of the diluted dye solution, and 1.5 ml. of acetone will be added. The pipetting and the addition of acetone require considerable care under these circumstances to avoid error.

[5] This can be obtained from the Macalaster Bicknell Company, Cambridge, Massachusetts. The screw shaft which is used to adjust the course of the syringe plunger must be filed to a point, and the surface of the syringe plunger must be flattened to a plane surface by grinding. In addition, it is necessary to mark a point on the end of the plunger so that it may be kept in line with a mark on the barrel, and thus used in the same position for delivery and calibration. Ordinary tuberculin type syringes may be used if modified as described.

[6] The requirements of plasma are: Two ml. unstained plasma, 1 ml. of which is used for the blank and 1 ml. for the standardization of plasma plus dye. Subsequent samples require only 1 ml. of plasma. Allowance must be made for the 1 ml. of whole blood required for a hematocrit. Knowledge of the approximate hematocrit in the patients permits economy of the amount of blood drawn.

then centrifuged at 2000 R.P.M. for 15 minutes. The precipitated proteins will be found packed at the bottoms of the tubes. The clear supernatants are transferred to three photometer cuvettes,[7] which are immediately stoppered to prevent evaporation of the acetone. At this point some protein particles may be found suspended in the acetone. If they do not settle in a few minutes they can be brought down by centrifugation for 5 minutes at 1500 R.P.M. Any air bubbles on the walls of the tubes will be removed at the same time.

The optical densities of the solutions are read in the spectrophotometer at $\lambda = 620$ mμ. The supernatants, while usually clear, have been found on occasion to be very faintly turbid. The turbidity has been the same in all tubes of a series and at most increases the optical density by 0.01. The turbidity increases if the tubes are cooled, and disappears if they are slightly warmed. It is desirable, therefore, to avoid any marked change in temperature during the whole procedure.

Calculations

An expression similar to that used by Phillips *et al.* (8) has been found convenient:[8]

$$\text{Plasma volume in ml.} = 5 \times V_R \times \frac{D_R - a}{D_V - a}$$

where D_R = optical density of the plasma to which dye has been added *in vitro*, D_V = optical density of the dye-stained plasma, a = optical density of the plasma blank, and V_R = volume in milliliters to which the dye in the syringe is diluted with saline.

The whole blood volume is calculated from the expression:

$$\text{Whole blood volume} = \frac{100 \times \text{plasma volume}}{100 - \text{true cell volume}}$$

where the true cell volume is found by multiplying by 0.955 the value found by the Wintrobe hematocrit[9] to correct for the 4.5 per cent of plasma trapped by the cells (9).

For calculation of the blood and plasma volumes by the falling curve method, the usual semilog plot of the optical density due to dye against time is used.

EXPERIMENTAL

Agreement with Beer's Law.—A series of experiments was done in which increasing amounts of the dye diluted 1:100 in 0.85 per cent saline were added to 1.0 ml. aliquots of plasma from different patients and normal individuals.

[7] Because centrifugation of the cuvettes may be necessary it is recommended that each investigator calibrate a set of 50 or so 12 × 75 mm. soft glass test tubes. Out of 200 such tubes it was possible to obtaine three sets of approximately 50 tubes, each set having an internal agreement of better than ± 1 per cent.

[8] The derivation (not given in reference 8) of this expression depends on the fact that the product of the concentration of the dye and the volume gives the total amount of dye present. If C_1 is the concentration of the dye in the reference solution, C_2 the concentration in the plasma, V_1 the volume of the reference solution, and V_2 the plasma volume, then $C_1 \times V_1 = C_2 \times V_2$ whence $V_2 = \frac{C_1 \times V_1}{C_2}$. In practice V_2 is made to be 1/5 V_1 so that only 0.2 ml. of the reference dye-NaCl solution need be added to the unstained plasma instead of the 1.0 ml. which would be required if V_1 were to equal V_2. Therefore V_R is multiplied by 5.

[9] The blood is pipetted into Wintrobe hematocrit tubes and centrifuged for 60 minutes at 3000 R.P.M. in a centrifuge with an 18 cm. radius.

The dye-stained plasmas were then carried through the acetone procedure. In all cases the optical density due to the dye was found directly proportional to the dye's concentration in the plasma. An example is given in Fig. 1. It was noted in the plasmas of patients with low protein that the amount of dye extracted was greater than in normal plasmas and tended to increase with decreasing protein concentration. A possible explanation of this effect is given later. With regard to the optical densities of the blank plasmas after treatment with acetone, the variation was so small compared to the optical density due to the dye that this variation could be neglected. For example five serial samples of blood taken from a normal individual after a low fat meal at 15

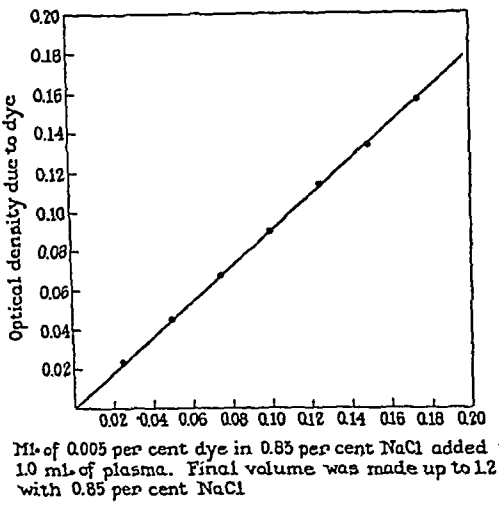

FIG. 1. Extinction-concentration curve of T-1824 in plasma treated with acetone.

minute intervals gave blank plasma optical densities of 0.081, 0.090, 0.071, 0.069, 0.068 by the standard method,[10] and of 0.009, 0.008, 0.008, 0.007, 0.008 after treatment with acetone.

Effect of Lipemia on the Acetone Method and the Standard Method[10].—The degree of lipemia had no effect on the amount of dye measured by the acetone method, as the fat particles disappeared on treatment with acetone. The optical densities of the supernatant acetone solutions from unstained plasmas

[10] The term "standard method" indicates the method in which the concentration of dye in plasma is estimated from the optical density of the dye-plasma mixture, without treatment with acetone. In the present work the standard method is applied as described by Phillips *et al.* (9) with the exception that smaller amounts of plasma are used, and that a separate reference solution of the dye in the unstained plasma of the subject is made as standard for each determination instead of using a single reference solution of the dye in a pooled clear plasma.

varied between 0.002 and 0.018, with the majority below 0.010. The optical densities of the original plasmas calculated to the same dilution varied from approximately 0.03 to 0.70. There was no correlation between the blank optical density and the degree of lipemia in the acetone method.

In an attempt to determine the effect of lipemia on the measurement of the optical density due to the dye in the standard method the following experiment was set up:—

Ten ml. of lipemic plasma from a nephrotic boy was centrifuged for 1 hour at 11,000 R.P.M. The separation into clear and turbid fractions was incomplete and only the upper, more

TABLE I

Effect of Lipemia on the Determination of T-1824 in Plasma by the Standard and Acetone Procedures

Plasma No.	Non-lipemic plasma	Lipemic plasma	0.005 per cent dye solution in 0.85 per cent NaCl	Sodium chloride 0.85 per cent	Standard procedure				Acetone procedure		
					Optical density with H_2O set at zero	Optical density due to dye	Optical density with plasma blank set at zero	Per cent of No. 2 determined	Optical density with H_2O set at zero	Optical density due to dye	Per cent of No. 2 determined
	ml.	*ml.*	*ml.*	*ml.*	*o.d.*	*o.d.*	*o.d.*	*per cent*	*o.d.*	*o.d.*	*per cent*
1	2.0	0.0	0.0	0.4	0.112				0.003		
2	2.0	0.0	0.4	0.0	0.745	0.633	0.640		0.173	0.170	
3	1.7	0.3	0.0	0.4	0.508	0.000			0.005		
4	1.7	0.3	0.4	0.0	1.155	0.647	0.668	104.3	0.175	0.170	100.0
5	1.3	0.7	0.0	0.4	0.915	0.000			0.009		
6	1.3	0.7	0.4	0.0	1.69±	0.775	0.680	106.2	0.180	0.171	100.6
7	1.0	1.0	0.0	0.4	1.120	0.000			0.013		
8	1.0	1.0	0.4	0.0	1.95±	0.830	0.795	124.3	0.185	0.172	101.2

The acetone procedure was carried out on aliquots of the plasmas examined by the standard procedure. The value obtained in No. 2 was taken as the theoretical under these conditions.

lipemic fraction was used (optical density approximately 2.0 at $\lambda = 620$ mμ). Duplicate mixtures were then made in which varying portions of this lipemic fraction were added to 1.0 ml. of non-lipemic plasma and the volume made up to 2.0 ml. with non-lipemic plasma from a normal individual. To each duplicate of one set was added 0.4 ml. of a dye solution containing 50 mg. of dye per liter of 0.85 per cent NaCl solution,[11] and to each of the other sets 0.4 ml. of 0.85 per cent NaCl solution. The optical density due to the dye was then estimated as closely as possible with both water and the appropriate plasmas without dye as blanks. (The densities of the lipemic plasmas were too great (Table I) for very precise measurements.) Then a 1.2 ml. aliquot of solution from each of the tubes was carried through the acetone procedure. From the results in Table I it can be seen that the most dense plasma with the standard method gave an error of + 24.3 per cent while the acetone method has an

[11] As noted by Gregersen and Stewart (10) T-1824 is not stable in 0.85 per cent NaCl. For this reason the 1:100 dilutions were made up fresh for each series of experiments that was carried out within the next few hours.

error of + 1.2 per cent, assuming that the dye concentrations found in mixtures of dye with non-lipemic plasma were exact. Similar results were obtained with plasmas of naturally different degrees of lipemia. Again the deviations from theoretical were greater in the more lipemic plasma, but it was not possible to make a general correction factor relating blank optical density and error. Similar experiments with trypan blue and methylene blue gave similar results. Briefly, then, in a lipemic plasma more dye is estimated by the "standard method" than is actually present. Examples of the extinction-concentration curves in normal and lipemic plasmas are given in Fig. 2. The relationship between percentage of dye present determined by the "standard method" and the optical density of the plasma blank is shown in Fig. 3 for various plasmas.

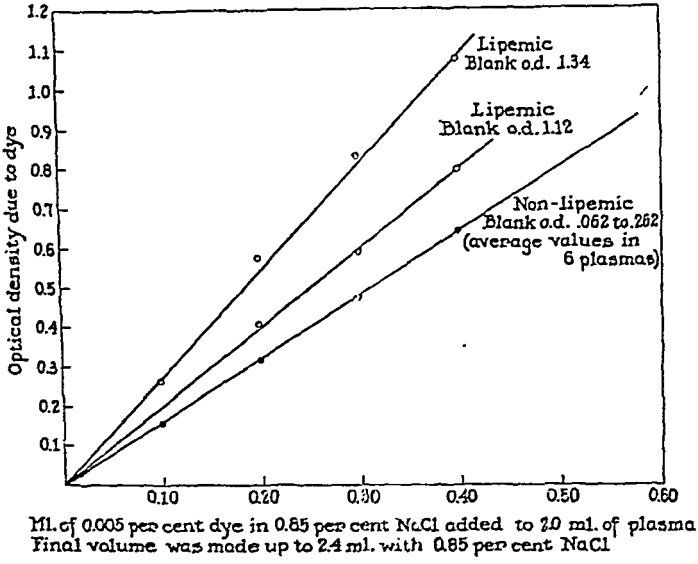

FIG. 2. Extinction-concentration curves of T-1824 in non-lipemic and lipemic plasmas with the standard method without acetone.

That the error encountered in analyses of lipemic plasma by the "standard method" was not attributable to the instrument was shown by the fact that similar results were obtained when duplicates were run with a Beckman spectrophotometer. In a lipemic plasma with an optical density of 1.34 the error with the Beckman, using 1.0 cm. cells, was + 75 per cent, and with the Coleman instrument + 72 per cent. The optical density of the same concentration of dye in a non-lipemic plasma was used as the true value.

A final proof that the lipemia was responsible for the error was furnished by the following experiment:

The same amount of dye was added to equal volumes of non-lipemic and lipemic plasmas (blank plasma optical densities 0.129 and 1.375 respectively). The amount of dye measured in the lipemic plasma was 159 per cent of that found in the normal plasma. The dye-stained

plasmas and their blanks were then centrifuged at 11,000 R.P.M. for 90 minutes (approximately 8,800 × *g*). The separation of the lipid particles was not complete, but the blank optical densities were reduced to 0.058 for the normal plasma and 0.210 for the lipemic plasma. It was therefore possible to apply the correction of Luetscher (2), which holds only for minor degrees of lipemia. With these corrections on both normal and lipemic plasmas (after centrifugation) the amount of dye measured in the lipemic plasma was 100.3 per cent of that found in the normal plasma, well within the limit of experimental error.

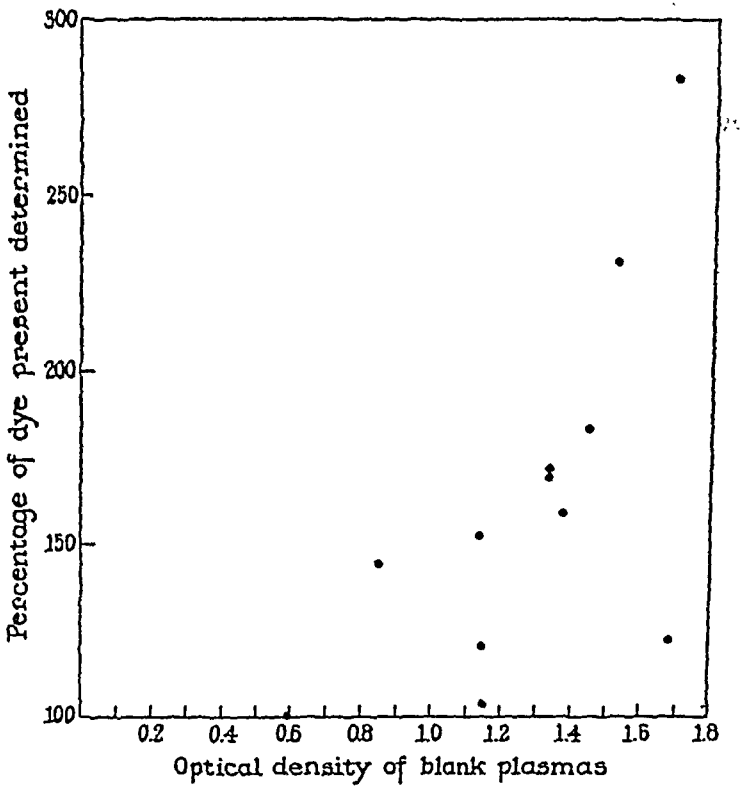

FIG. 3. Effect of lipemia on the percentage of dye present determined by the standard method.

Effect of Hemolysis.—The effect of hemolysis was examined by adding 0.1 ml. of a hemoglobin solution, obtained by laking 3 ml. of red cells with 97 ml. of distilled water and filtering twice, to aliquots of four different plasmas to which dye in some cases and 0.85 per cent NaCl in others had been added (see Table II). A portion of plasma with no added hemoglobin was used as the blank. After the acetone treatment it was found that the variation was approximately ± 3 per cent of the amount of dye that was measured in the same plasmas without adding hemoglobin. The amount of hemoglobin involved was sufficient to cause gross coloration of the plasma to which it was added. Similar experiments with smaller amounts of added hemoglobin gave smaller errors. With

10 times as great a hemoglobin concentration, the error was larger but did not exceed ± 3.5 per cent; in these cases, however, the amount of hemoglobin present in the plasmas not treated with acetone was so great that the very high optical densities of both blank and dye-stained plasmas entirely precluded using Gibson and Evans' (11) correction for hemoglobin with the standard method.

TABLE II

Effect of Added Hemoglobin on the Determination of T-1824 in Plasma with Acetone

Plasma No.		*a* Plasma	*b* 0.005 per cent dye solution in 0.85 per cent NaCl	*c* Sodium chloride 0.85 per cent	*d* Hemoglobin solution (3 ml. red cells laked in 97 ml. distilled H_2O)	*e* Optical density with H_2O set at zero	*f* Optical density due to dye with appropriate blank set at zero $a' - b'$ $c' - d'$
		ml.	*ml.*	*ml.*	*ml.*	*o.d.*	*o.d.*
1	a′	1.0	0.1	0.1	0.0	0.094	0.092
	b′	1.0	0.0	0.2	0.0	0.002	
	c′	1.0	0.1	0.0	0.1	0.096	0.093
	d′	1.0	0.0	0.1	0.1	0.003	
2	a′	1.0	0.1	0.1	0.0	0.092	0.086
	b′	1.0	0.0	0.2	0.0	0.006	
	c′	1.0	0.1	0.0	0.1	0.095	0.086
	d′	1.0	0.0	0.1	0.1	0.009	
3	a′	1.0	0.1	0.1	0.0	0.118	0.100
	b′	1.0	0.0	0.2	0.0	0.018	
	c′	1.0	0.1	0.0	0.1	0.115	0.102
	d′	1.0	0.0	0.1	0.1	0.013	
4	a′	1.0	0.1	0.1	0.0	0.108	0.104
	b′	1.0	0.0	0.2	0.0	0.004	
	c′	1.0	0.1	0.0	0.1	0.110	0.104
	d′	1.0	0.0	0.1	0.1	0.006	

Optical densities of plasmas before treatment with acetone were for No. 1, 0.088, No. 2, 0.134, No. 3, 1.50, No. 4, 1.10. The hemoglobin was added to the plasma *after* the dye had been added.

Comparison of Results Obtained with Standard and Acetone Methods.—In the cases listed in Table III, the standard method was first carried out, then 1.2 ml. aliquots of each sample of plasma were taken through the acetone procedure and a reference solution in dye-free plasma was made in each case. The results have been grouped according to the degree of lipemia; Nos. 1 through 14 are essentially clear, Nos. 15 through 21 are lipemic to a varying degree, indicated by the optical densities of the plasma blanks. For the non-lipemic plasmas,

the mean of the plasma volume measured by the acetone method is 99.46 per cent of the mean by the standard method, with an estimated standard deviation of ± 1.97 per cent. Gross deviations occurred in the lipemic plasmas. In many lipemic cases it was not possible to determine the plasma volume by the

TABLE III

Comparison of Plasma Volume Determinations by the Standard and Acetone Procedures

Plasma No.	Patient	Optical density of blank plasma by standard procedure	Plasma volume determined by		$\frac{100 \times b}{a}$	$b - a$
			Standard procedure *a*	Acetone procedure *b*		
		o.d.	*liters*	*liters*		*liters*
1	A.C.	0.062	2.29	2.33	101.7	+0.04
2	M.B.	0.080	2.56	2.52	98.5	−0.04
3	M.B.	0.080	2.59	2.55	98.4	−0.04
4	M.B.	0.080	2.64	2.57	97.4	−0.07
5	A.H.	0.087	2.27	2.28	100.4	+0.01
6	A.M.	0.091	2.63	2.56	97.3	−0.07
7	P.R.	0.091	1.09	1.06	97.2	−0.03
8	P.R.	0.100	1.39	1.35	97.2	−0.04
9	R.A.	0.117	2.47	2.54	102.8	+0.07
10	W.K.	0.148	3.57	3.61	101.1	+0.04
11	A.M.	0.149	2.53	2.46	97.2	−0.07
12	A.M.	0.159	2.44	2.45	100.4	+0.01
13	A.M.	0.159	2.39	2.45	102.4	+0.06
14	R.A	0.291	2.62	2.63	100.4	+0.01
15	M.B.	0.229	2.25	2.10	93.4	−0.15
16	P.R.	0.282	1.15	1.09	94.4	−0.06
17	A.F.	0.290	1.27	1.20	94.5	−0.07
18	M.M.	0.382	3.25	3.01	92.7	−0.24
19	C.C.	0.387	1.19	1.25	104.4	+0.06
20	M.M.	0.436	3.30	2.94	88.8	−0.36
21	P.R.	0.638	1.30	1.12	86.2	−0.18

Nos. 2, 3, 4, 12, and 13 were calculated from serial samples obtained for a falling curve plot. Nos. 12, 13, and 14 were not lipemic but contained residual dye from previous determinations.

standard method because of the extremely high plasma blanks. With the exception of these omissions, the table gives the results of consecutive determinations.

Effect of Protein Concentration in Plasma on Amount of Dye Extracted by Acetone.—Plasmas from normal individuals were found to yield somewhat smaller recoveries of added dye than did plasmas from patients with hypoproteinemia. Since T-1824 is known to be bound to albumin in plasma (12) it was considered desirable to determine the effect of albumin concentration on the

recovery of the dye. For this purpose a plasma containing 2.20 gm. of albumin per 100 cc. (total protein 4.10 gm. per 100 cc.) was chosen, and to aliquots were added increasing amounts of human plasma albumin. T-1824 was found to be adsorbed by the albumin when the proteins were precipitated by acetone. It was found, as shown in Table IV, that less dye was recovered in the acetone extract as the concentration of albumin in the plasma was increased; the recovery was 95 per cent from plasma that had 2.0 gm. of albumin per 100 cc. and 85 per cent from plasma that had 4.0 gm. (Table IV). The T-1824 adsorbed by the precipitated protein could not be readily recovered by repeated extraction of the precipitate with acetone.

TABLE IV

Effect of Added Albumin on Extraction of T-1824 by Acetone

Solution No.	Concentration of albumin	Optical density due to dye
	gm. per 100 cc.	*o.d.*
1	2.20	0.196
2	2.83	0.190
3	3.50	0.185
4	4.34	0.174
5	5.17	0.163
6	6.00	0.156

No. 1 is the plasma of a nephrotic patient with an optical density of about 1.6 with the standard procedure. Nos. 2 through 6 are samples of this plasma to which human albumin was added in sufficient amounts to bring the albumin concentration to the level indicated. The same amount of dye was present in all the plasmas.

Addition of γ globulin (immune serum globulin) to normal and hypoproteinemic plasmas had a similar effect on the percentage of dye extracted by acetone.

Error from adsorption of dye by the proteins is avoided in the acetone method by preparing the reference solution in each case from a mixture of the dye and the subject's previously drawn plasma, so that adsorption is the same in the reference mixture as in the analysis of the plasma drawn after dye infusion.

Non-Applicability of the Acetone Method to Whole Blood

When the acetone method was applied to whole blood a clear filtrate was obtained, in which the dye concentration was proportional to that in the whole blood, in the case of a given blood. However, about half of the dye was adsorbed by the hemoglobin precipitate. The adsorbed fraction varied with the cell content of the blood, and the dye concentrations of the filtrates were too low (about one-quarter as much as in plasma filtrates) for optimal accuracy of measurement in a Coleman photometer.

DISCUSSION

The principle of using a solvent which will precipitate proteins, dissolve lipids, and extract dye, is not new in its application to the determination of dyes in lipemic plasmas. Among others, Dragstedt and Mills (13) have used acetone in a ratio of 2:1 to plasma for the determination of bromsulfalein. Taran (14) has used acetone in a 1:1 ratio to plasma for the Congo red test for amyloidosis, and Ernst and Förster (15) have employed a similar method for bilirubin. More recently with brilliant vital red Robinow and Hamilton (16) in blood volume studies in infants found 95 per cent ethyl alcohol satisfactory, though approximately one-third of the dye was adsorbed by the protein precipitate formed; with T-1824 these authors found that 75 per cent of the dye was precipitated. Acetone was dismissed because it evaporated too rapidly.

Before acetone was adopted in the present procedure many other substances were tried in an attempt to achieve the results obtained with acetone. Dioxane, methyl cellosolve, collidine, *n*-butanol, *n*-propanol, isopropanol, ethanol, methanol, and anionic and cationic detergents, were all tried unsuccessfully. A series of preliminary experiments with methanol in the same proportions as the acetone seemed at first to be quite promising. They were abandoned because the hemoglobin in plasma from partly hemolyzed blood adsorbed relatively large amounts of dye when the proteins were precipitated with methanol, and caused large errors in the measurement of the dye. In addition it was found necessary to cool the plasmas to which the methanol had been added for 1 hour at 4°C. to complete the flocculation of denatured proteins. Acetone alone was found to be relatively insensitive to the presence of hemolysis and to be adequate for the solution of the lipids and extraction of the T-1824.

The protein concentration in the plasma examined is apparently the determining factor in the percentage of the dye extracted from plasma by acetone. This necessitates establishing a standard calibration curve for each individual, and for each concentration of protein when the protein concentration of the subject's plasma is changed as the result of infusions or otherwise. However, neglect of this precaution would cause relatively small errors only when the protein changes are small.

If one assumes, as have Le Veen and Fishman (17), that the dye protein combination is a reversible one, then the simplest formulation is

$$P + D \rightleftharpoons PD \tag{1}$$

with

$$S = [D] + [PD] \tag{2}$$

where P is protein, D is free dye, PD is dye-protein combination, and S is total amount of dye in system. Then

$$\frac{[P][D]}{[PD]} = K \tag{3}$$

whence, combining (2) and (3) we obtain

$$[D] = \frac{KS}{K + [P]}. \tag{4}$$

Here, the concentration of free dye, $[D]$, will depend not only on the total amount of dye present S, but also on $[P]$. However, if $[P]$ is held constant, and it is under these circumstances that the acetone method has been used, then (4) reduces to $[D] = K'S$ and the amount of free dye is determined, with $[P]$ constant, solely by the total amount of dye S. This is adequately demonstrated in Fig. 1. If one were to attempt a semilog plot for a falling curve of dye in plasma in the face of rapidly shifting protein concentrations it would be necessary to determine K in equation (4) for each concentration of protein.

The results reported here do not necessarily contradict those of Gregersen (18), who compared the amounts of dye estimated in non-lipemic plasmas with the amounts of dye estimated in lipemic plasmas from the same individuals after they had taken a fatty meal. He found no difference in the amounts of dye estimated. However, in our experience, the lipemia found after meals in normal individuals has never equalled the extremes of lipemia found in nephrotic individuals. Further, at blank optical densities up to 1.0, the errors in the amount of dye estimated by the standard method are not necessarily very great.

The effect of the lipemia on the determination of the extinction due to dye can best be discussed by referring to the optical properties of sols or suspensions in general. It is generally accepted that Beer's law holds for dilute suspensions. However, in concentrated suspensions deviations from Beer's law are found; the optical density increases less rapidly than the concentration of the suspension. (See for example the work of Lange (19), Wells (20), Dreosti (21), and of Bloch and Renwick (22).) Wells suggested that some of the light scattered in dense suspensions finds its way back into the original beam after multiple reflections in every which way among the particles. This secondary scattering has been well recognized, but it has not been so well recognized that the effect of the suspensions in a dye solution is to permit passage of light through dye molecules which would not be in the normal path of light. Lipemia, then, acts as if it increased the length of the path of light through the cuvette. This results in an erroneously high value for the optical density due to the dye. (Gibson, quoted by Thorn, was apparently the first to draw attention to the error in lipemic plasmas (1).) This error will depend, naturally, on the degree of lipemia, and the greater the lipemia the greater will be the apparent optical density due to the dye, as shown by the extinction-concentration curves of Fig. 2 and the points in Fig. 3. Fig. 3, in addition, shows that the error in the determination of the dye is not necessarily the same in plasmas of the same optical density. That the error is entirely due to the lipemia is demonstrated by the experiment in which the error disappeared after centrifuging out the lipid particles.

Three salient facts must therefore be emphasized. (*a*) At low particle concentrations, the degree of lipemia is directly proportional to the optical density, and corrections for changes in optical density due to lipemia may be made as described by Luetscher (2). In addition, the extinction-concentration curves for dye in plasma will be identical in different plasmas and the specific extinction of the dye in a pooled plasma may be used to determine the concentration of the dye in other plasmas suitable corrected for the plasma blank, provided the lipemia is of a minor degree. (*b*) At high particle concentrations, deviations from Beer's law occur, more of the dye is estimated than is actually present, the extinction-coefficient is not the same in all plasmas even of the same blank optical density, and Luetscher's correction for changes in degree of lipemia cannot be applied. (*c*) If the standard method is used and the extinction of the dye is determined in the lipemic plasma, the results are likely to be inaccurate because the plasma used for the reference standard and the plasma of unknown dye content may vary in degree of lipemia even when the two plasmas are drawn within a few minutes from the same subject.

A word may be said here about the particles responsible for the lipemia. It has been found (*e.g.* Fig. 2) that for a given lipemic plasma Beer's law is roughly obeyed for the dye, but the deviation from a straight line may be as high as ± 5 per cent. This suggests that the lipid suspensions in plasmas are not entirely stable, and that the instability may be one of the reasons for the erratic results obtained with the standard method. If the plasmas are allowed to stand, particularly in the cold, some of the lipid particles will coalesce, thus introducing unpredictable and uncorrectable errors. In addition, in some plasmas, centrifugation at 3,000 R.P.M. for 15 minutes has been sufficient for the formation of a "butter" layer at the top.

While the method has been described for small amounts of plasma, there is no apparent reason why, when larger amounts of plasma are available, all additions cannot be increased proportionately. This would permit the use of larger cuvettes which, giving higher optical densities, might decrease the experimental error.

SUMMARY

A method is presented for estimating the concentration of T-1824 in lipemic plasma. The plasma is mixed with acetone, which precipitates the proteins, dissolves or precipitates the lipids, and yields a clear solution for photometric measurement of the dye. The method is not sensitive to changes in the degree of lipemia and is relatively insensitive to hemolysis.

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THE EFFECT OF NITROGEN MUSTARDS ON ENZYMES AND TISSUE METABOLISM*

I. The Effect on Enzymes

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The halogenated alkylamines, so called nitrogen mustards, investigated during the last war as a potential warfare agent, have become useful tools in biology and medicine. Possessing some of the properties of bis(β-chloroethyl) sulfide, and x-rays (such as injurious effects to the bone marrow and lymph glands which lead to leucopenia (1), inhibition of mitosis (2, 3), and production of mutants (3–5), they are being used with success in the treatment of blood discrasias (6–8). Some of the studies on the chemical properties of the nitrogen mustards have been published (9–14). We present in this paper experiments performed in this laboratory during the years 1942–43, and quite recently on the effect of nitrogen mustards on the activity of enzymes. These studies were undertaken to determine their mechanism of action. Nitrogen mustards were found to be powerful enzyme inhibitors belonging to the class of structural inhibitors, although different from most of the compounds of this group in the lack of easy reversibility and competition.

Nitrogen Mustards and Enzymes

A number of investigators (9–15) have shown that tertiary halogenated alkylamines when in aqueous neutral solutions undergo a series of rapid transformations, the first of which is the formation of the ethylenimonium ring. In dilute solutions, according to Cohen (15), this first change is a rapid process while the rearrangement which ends with the formation of the dimer or the ethanolamine proceeds at a rate 100 times as slow as the rate of the first process. It is therefore reasonable to assume that in the first hour after the compounds have been brought into aqueous solutions, at pH values around neutrality,

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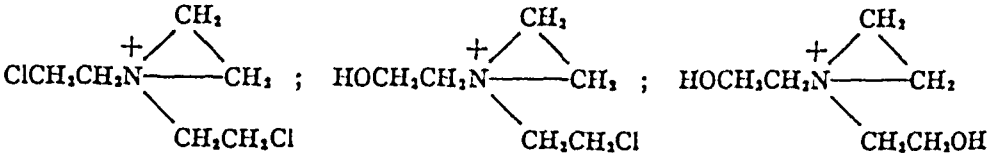
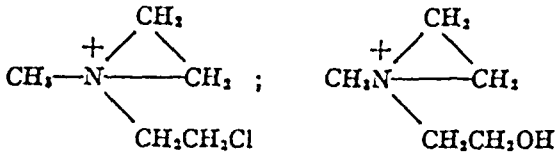
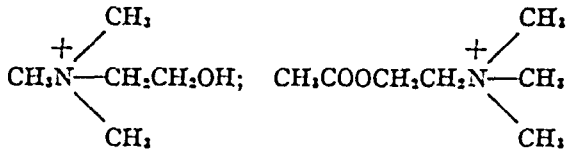
there are present at different concentrations three different ethylenimonium compounds:



in the case of tris(β-chloroethyl)amine; two compounds:



in the case of methyl-bis(β-chloroethyl)amine. The resemblance of these transformation products to choline and acetylcholine is striking:



Choline Acetylcholine

This similarity between the ethylenimonium derivatives of nitrogen mustards and choline and acetylcholine suggested the possibility that they may act as structural inhibitors of enzymes by combination with the protein moiety at the side chains where combination of choline and acetylcholine takes place. The validity of this assumption is demonstrated in the experiments with choline oxidase, acetylcholine esterase, and choline acetylase.

1. Choline Oxidase.—In agreement with the postulated assumption, choline oxidase was, of all the enzymes studied, the most sensitive to the action of nitrogen mustards. In these experiments choline oxidase was prepared from rat liver by a modification of the method of Bernheim (16); the enzyme suspension consumed no oxygen in the absence of choline. When methyl-bis-(β-chloroethyl)amine HCl (MBA) and tris(β-chloroethyl)amine HCl (TBA) were dissolved in phosphate buffer, 0.02 M pH 7.4, the degree of inhibition remained constant up to 2 hours after solution of the compound with TBA, while it decreased rapidly with MBA. When the compounds were dissolved in water, inhibition increased on standing (Table I). Isopropyl-bis(β-chloroethyl)amine HCl was also found to be a powerful inhibitor of choline oxidase (Table II). Since formation of the ethylenimonium derivative occurs more rapidly in phosphate buffer than in water (17), these experiments indicate that inhibition was due to the quaternary N derivative. Since inhibition disappeared when MBA was left standing in phosphate buffer for 2 hours, and TBA for 24 hours, it must be concluded that the end products of the series of reactions

TABLE I

Inhibition of Choline Oxidase by Nitrogen Mustards. The Effect of Standing in Aqueous and Phosphate Solutions

Methyl-bis(β-chloroethyl)amine HCl (MBA) and tris(β-chloroethyl)amine HCl (TBA) were dissolved and added to the enzyme suspension after remaining in solution for the time indicated in Table I.

Time of standing	MBA	Inhibition	TBA	Inhibition
min.	*M*	*per cent*	*M*	*per cent*
In phosphate buffer				
0	1×10^{-5}	95	1×10^{-5}	74
15	"	72	"	79
30	"	38	"	84
60	"	13	"	74
120	"	0	"	75
1440	"	0	"	0
In water				
0	"	42	"	34
30			2.5×10^{-6}	40
60	5×10^{-7}	32		
90			"	57
120	"	42	1×10^{-5}	74
210			2.5×10^{-6}	62
480	"	76		

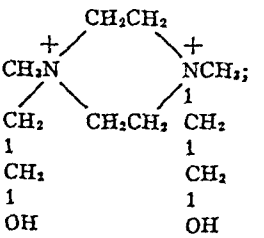
TABLE II

Inhibition of Choline Oxidase by Isopropyl-Bis(β-Chloroethyl)amine HCl (IBA)

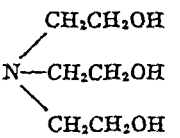
Concentration of choline, 0.02 M. pH, 7.4. Temperature 38°.

IBA	Inhibition
M	*per cent*
7×10^{-5}	Complete
3×10^{-5}	97
1×10^{-5}	44
5×10^{-7}	22

which nitrogen mustards undergo in water solutions, namely the dihydroxy cyclic dimer (I) or tri-ethanolamine (II), have no inhibitory power.



I



II

Further evidence that the inhibition is produced by the ethylenimonium transformation products is given by experiments in which the inhibitory power of nitrogen mustards was measured at different pH values. If the inhibition is produced by these quaternary N derivatives, it must increase as the pH values increase because the rates of transformation are so affected (15). In fact, 2×10^{-6} M TBA, which produced 68 per cent inhibition at pH 6.4, inhibited completely at pH 7.4; 1×10^{-7} M produced 15 per cent inhibition at pH 7.4, while it increased to 36 per cent at pH 8.4. Similar results were obtained with MBA (Table III).

It seems that inhibitors belonging to this group must be able to form the ethylenimonium derivative. β-chloroethylethylbenzylammonium chloride, β-chloroethylethylcyclohexylammonium chloride, and β-chloroethylethyl hexahydrobenzylammonium chloride[1] which form ethylenimonium derivatives

TABLE III

Effect of pH on the Inhibition of Choline Oxidase by Alkylamines

Phosphate buffer. Temperature 38°.

pH	Methyl-bis(β-choroethyl)amine		Tris(β-chloroethyl)amine	
	Concentration	Inhibition	Concentration	Inhibition
	M	*per cent*	M	*per cent*
6.4	2×10^{-6}	68	2×10^{-6}	None
7.4	1×10^{-7}	15	2.5×10^{-6}	42
	2×10^{-6}	Complete	1×10^{-6}	27
8.4	1×10^{-7}	36	"	81

on solution in water were as powerful inhibitors as MBA. The quaternary N compound dimethyl-bis(β-chloroethyl)amine HCl which does not form ethylenimonium on solution had no inhibitory effect. Trimethylbenzylammonium chloride, dimethylethanolamine, and methylethanolamine had no effect at all (Table IV).

One important difference between nitrogen mustard inhibition and other substrate inhibitors (such as the classical malonate inhibition of succinoxidase discovered by Quastel and Wooldrige (18)) is that the former is not, like the latter, easily reversed, and as a consequence does not depend on the ratio of substrate to inhibitor. Once inhibition was produced with the nitrogen mustards it was difficult to reverse, and washing the enzyme with water brought no restoration of activity. This strong association of the enzyme with the nitrogen mustard in contrast with the reversible association with choline can be shown by measuring the half-saturation of the complex enzyme-choline and half-

[1] These compounds were kindly provided by Dr. Mark Nickerson, Department of Pharmacology, University of Utah, School of Medicine.

inhibition of enzyme activity. Half-saturation (Fig. 1) was reached with 2.5×10^{-3} M choline while half-inhibition was reached with 1×10^{-6} M TBA, 4×10^{-6} M MBA, and 1.2×10^{-6} M isopropyl-bis(β-chloroethyl)amine HCl.

TABLE IV

Effect of Some Ethylenimonium-Forming Compounds on the Activity of Choline Oxidase

Phosphate buffer, pH, 7.4. Choline, 0.01 M. Duration of experiments, 1 hour. Temperature 38°.

Inhibitor	Concentration	O_2 uptake	Inhibition
		c.mm.	*per cent*
None	—	205	—
MBA	5×10^{-6}	41	80
β-Chloroethylethylbenzylammonium chloride	"	46	78
β-Chloroethylethyl hexahydrobenzylammonium chloride	"	10	95
β-Chloroethylethylcyclohexylammonium chloride	"	9	95

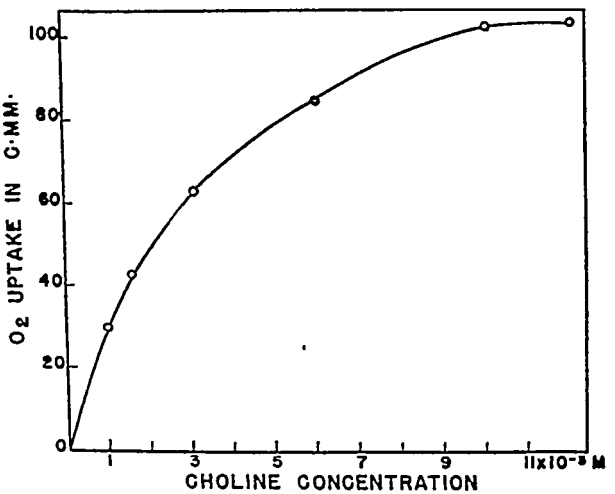


FIG. 1. Effect of choline concentration on the oxidation of choline by choline oxidase pH 6.7. Abscissa, choline concentration $\times 10^{-3}$ M. Ordinate O_2 uptake in 10 minutes. Temperature 38°.

Since the LD_{50} values of nitrogen mustards lie around 2 to 5×10^{-5} M, it can be seen that complete inhibition of choline oxidase occurred with amounts ten times lower.

Indication that the nitrogen mustards combine with the protein moiety of the enzyme (activating protein or dehydrogenase) at the place where combination with choline takes place, was given by experiments where choline was

added previous to or simultaneously with the nitrogen mustards, and by experiments where large amounts of choline were added after addition of nitrogen mustard to the enzyme. When choline was added to the enzyme previous to addition of MBA, or when choline and MBA were added simultaneously, there was no inhibition. When 2×10^{-2} M choline was added to the enzyme after addition of MBA, the inhibition decreased from 80 per cent to 41 per cent; on increasing the concentration of choline to 1×10^{-1} M the inhibition was further lowered to 35 per cent (Table V).

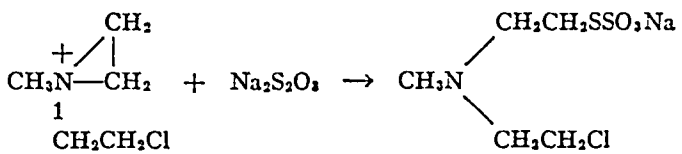
TABLE V

Competition between Choline and MBA (10^{-6} M) for the Activating Protein of Choline Oxidase

Choline concentration, 1×10^{-2} M. pH, 6.7. Temperature 38°.

Experiment	O_2 uptake		Inhibition
	Control	Inhibitor	
	c.mm.	*c.mm.*	*per cent*
MBA added to enzyme 15 min. before choline addition	320	64	80
MBA + enzyme mixed. Choline, 2×10^{-2} M added later	344	202	41
Choline, 10^{-1} M	309	210	35
MBA + choline added simultaneously	315	310	None
Choline + enzyme mixed. MBA added later	315	300	4

Thiosulfate reacts very rapidly with the ethylenimonium derivative of nitrogen mustards; *e.g.*, MBA:



Addition of thiosulfate (1×10^{-3} M) to choline oxidase previous to addition of MBA (1×10^{-5} M) prevented inhibition. When thiosulfate was added 5 minutes after contact of the enzyme with the nitrogen mustard, the inhibition was one-third of the original (Table VI). A number of substances known to combine rapidly with nitrogen mustards were tried to prevent enzyme inhibition. The inhibitor and the test substance were added to Ringer-phosphate, pH 7.4, and were allowed to react for 15 minutes, at the end of which they were added to the enzyme suspension. Of all the compounds tested, only thiosulfate had a significant preventive effect. A concentration of thiosulfate 1000 times greater than the concentration of nitrogen mustard brought about complete prevention of inhibition; when the ratio was 100:1 the prevention

dropped to 50 per cent; with a ratio of 10:1 there was no prevention at all. Reversal of inhibition with the preventive agents did not occur (Table VII). Tryptophane, tyrosine, inositol, cysteine, and histidine had no preventive effect.

2. Acetylcholine Esterase.—The inhibitory effect of methyl-bis(β-chloroethyl)-amine HCl on acetylcholine esterase was first observed by Thompson (19), who also described prevention of inhibition if acetylcholine had been added previously. Thompson's experiments were confirmed, as can be seen in Table VIII. If nitrogen mustards act as structural inhibitors, they must have no effect or very little on the hydrolysis of other esters. In fact, the hydrolysis of acetylcholine by brain choline esterase was inhibited 70 per cent by 1×10^{-3} M MBA, while the hydrolysis of monobutyrin was not affected at all. With purified serum esterase, hydrolysis of acetylcholine was 40 per cent inhibited while hydrolysis of monobutyrin was inhibited 13 per cent (Table IX).

Thiosulfate prevented the inhibition. When it was added 5 minutes after addition of TBA, there was partial reversal (Table X).

TABLE VI

Effect of Thiosulfate on the Inhibition of Choline Oxidase by Tris(β-Chloroethyl)amine HCl (TBA)

System	O_2 uptake	Inhibition
	c.mm.	*per cent*
Choline	234	—
" + 10^{-6} M TBA	58	75
" + 10^{-3} M $Na_2S_2O_3$	211	—
" + 10^{-3} M $Na_2S_2O_3$ + 10^{-5} M TBA added 15 min. later	188	11
" + 10^{-4} M $Na_2S_2O_3$ + 10^{-5} M TBA	80	58
" + 10^{-5} M TBA + 10^{-3} M $Na_2S_2O_3$ added 5 min. later	153	27

TABLE VII

Inhibition of Choline Oxidase by Methyl-Bis(β-Chloroethyl)amine HCl (MBA) (1×10^{-5} M) and Its Prevention by Antidotes

Antidote	Concentration	Reactivation
	M	*per cent*
$Na_2S_2O_3$	10^{-3}	90
$Na_2S_2O_3$	10^{-4}	52
$Na_2S_2O_3$	10^{-5}	0
BAL	10^{-3}	30
Urotropin	"	21
"	10^{-4}	0
Glutathione	10^{-3}	17
Thiourea	"	17
"	10^{-4}	0

3. Choline Acetylase.—Another reaction in which choline is one of the reactants is the synthesis of acetylcholine. Feldberg (20) reported that this reaction was inhibited by methyl-bis(β-chloroethyl)amine. This has been confirmed

TABLE VIII

Effect of Nitrogen Mustards on the Hydrolysis of Acetylcholine by Serum and Brain Esterases

In Ringer-bicarbonate (N_2:CO_2 as gas phase) pH, 7.4. Temperature 25°.

System	TBA	Inhibition	MBA	Inhibition
	M	*per cent*	*M*	*per cent*
Rat brain				
Inhibitor freshly prepared	1×10^{-4}	81	1×10^{-4}	61
2 hrs. after	"	32.5	"	69.5
6 " "	"	22	"	65
Horse serum				
Purified enzyme Inhibitor, fresh.	5×10^{-4}	73.4	1×10^{-3}	16.5
2 hrs. after	"	20.7	"	17.0
Human serum				
Inhibitor, fresh	1×10^{-4}	63.9	5×10^{-4}	46.6
2 hrs. after	"	13.2	"	41.7
	1×10^{-3}	Complete	1×10^{-3}	88
	1×10^{-4}	54	1×10^{-4}	26
Chicken serum	1×10^{-4}	58.4	"	27
	5×10^{-4}	Complete	5×10^{-4}	50.8
Chicken brain	1×10^{-4}	57.5	1×10^{-4}	32
Inhibitor in H_2O, fresh	5×10^{-4}	80	5×10^{-4}	93
" " " at 6 hrs. later.	"	56	"	92

TABLE IX

Effect of Methyl-Bis(β-Chloroethyl)amine HCl (MBA) (0.001 M) on the Activity of Esterases

Buffer, Ringer-$NaHCO_3$ plus 3 cc. of 0.1 M $CaCl_2$ added to 100 cc. Saturated with N_2:CO_2. pH, 7.5. Substrate, 0.01 M. Duration of experiment, 20 minutes. Temperature, 38°.

Experimental conditions	Serum esterase		Brain esterase	
	CO_2 output	Inhibition	CO_2 output	Inhibition
	c.mm.	*per cent*	*c.mm.*	*per cent*
Acetylcholine	146		344.5	
" +MBA	87.4	40	102.9	70
Monobutyrin	124.8		131.5	
" +MBA	108	13	130.8	None

in experiments in which acetylcholine synthesis was measured in acetone-dry brain extracts, anaerobically, and in the presence of citrate, adenosinetriphosphate, choline, and boiled yeast extract. Synthesis of acetylcholine was inhibited 70 per cent by 1×10^{-3} M of MBA.

4. Effect of Nitrogen Mustards on Other Enzymes.—Nitrogen mustards in aqueous, weakly alkaline solutions are very reactive. They react rapidly with —SH groups like cysteine, glutathione, and the —SH groups of denatured albumin, removing half of the —SH groups in 5 minutes (21, 22). They react (pH 8) with a large number of organic compounds of biological importance (NH_2 groups and carboxyl groups of amino acids, peptides, and proteins, nicotinic acid, methionine, thiamine, adenylic acid, adenosinetriphosphate, pyridoxine, P organic compounds) (13, 14)), some of them essential for enzyme activity.

TABLE X

Effect of Thiosulfate on the Inhibition of Choline Esterase by Tris(β-Chloroethyl)amine HCl

Concentration, 2.5×10^{-4} M; thiosulfate, 2.5×10^{-2} M. Temperature 38°. Time of experiment, 60 minutes.

System	CO_2 output	Inhibition
	c.mm.	*per cent*
Enzyme + AC*	146.1	
" + AC + $Na_2S_2O_3$	139.3	5
" + TBA; AC from side arm	18.7	87
" + AC; TBA " " "	126.5	9.6
" + AC + TBA. $Na_2S_2O_3$ 5 min. later	71.1	51
" + AC + $Na_2S_2O_3$. TBA 5 " "	132.7	9

* AC, acetylcholine.

TABLE XI

Effect of Methyl-Bis(β-Chloroethyl)amine HCl on P Exchange Enzymes

Enzyme	MBA	Inhibition	Investigator
	M	*per cent*	
Phosphocreatine phosphokinase	5×10^{-3}	75	Cori *et al.*
Reduced state	4×10^{-3}	84	" " "
Oxidized "	"	33	" " "
Phosphopyruvate phosphokinase	"	42	" " "
Adenosinetriphosphatase (myosine)	1×10^{-3}	None	Barron *et al.*
Adenosinetriphosphatase	4×10^{-3}	26	Cori *et al.*
Myokinase	"	None	" " "
Inorganic pyrophosphatase	1×10^{-3}	67	" " "
Reduced state	4×10^{-3}	80	" " "
Oxidized "	"	87	" " "
Hexokinase	2.5×10^{-3}	65	Dixon
"	4×10^{-3}	37	Cori *et al.*
Deuterohexokinase	1×10^{-2}	55	" " "
Acid phosphatase	1×10^{-3}	40	Barron *et al.*
Alkaline "	"	8	" " "

This high reactivity of the halogenated alkylamines would make them powerful inhibitors of a large number of enzymes. However, experiments performed by a number of investigators (Peters *et al.* and Dixon *et al.* in England, Cori and this laboratory in the United States) do not support this contention. In fact, if *in vitro* experiments with concentrations above 10 times the lethal

TABLE XII

Effect of Nitrogen Mustards (0.001 M) on the Activity of Some Enzyme Systems. (Other than P Exchange Enzymes)

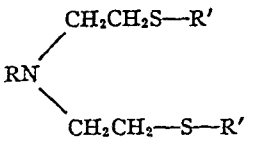
Enzyme	Substrate	Determination	Inhibition		Investigators
			TBA	MBA	
			per cent	*per cent*	
Choline oxidase	Choline	O_2 uptake	Complete	Complete	Barron *et al.*
Choline esterase	Acetylcholine	Acid formation, CO_2	"	"	Thompson, Barron *et al.*
" "	"	" " "	"	"	Barron *et al.*
Betaine aldehyde oxidase	Betaine aldehyde	O_2 uptake	"	"	" " "
Pyruvate oxidase	Pyruvate	" " and pyruvate utilization	"	"	" " "
Adenosine deaminase	Adenosine	NH_3 formation	None	None	" " "
Uricase	Uric acid	O_2 uptake		27	" " "
Phosphoglyceraldehyde dehydrogenase	Phosphoglyceraldehyde	DPN reduction	None	None	" " "
Polyphenol oxidase	Catechol	O_2 uptake	"	"	" " "
Carbonic anhydrase	$NaHCO_3$	CO_2 output	"	"	" " "
Enolase			"	"	Cori *et al.*
Isomerase			"	"	" " "
Phosphoglucomutase			"	"	" " "
Lactic dehydrogenase	Lactate	DPN reduction	"	"	Barron *et al.*
Succinic oxidase	Succinate	O_2 uptake	"	"	" " "
Cytochrome "	Cytochrome C	Reduced cytochrome C oxidation	"	"	" " "
Diamine "	Histamine	O_2 uptake	"	"	" " "
Transaminase	Glutamate + pyruvate	Alanine	"	"	" " "
Carboxylase	Pyruvate	CO_2 output	"	"	" " "
Arginase	Arginine	NH_3 formation	"	"	" " "
Pepsin	Hemoglobin	Tyrosine	6	6	" " "
Trypsin	"	"	11	11	" " "
Papain	"	—		None	Dixon
d-Amino acid oxidase	Alanine	Pyruvate	26	"	Barron *et al.*

dose (about 1×10^{-4} M) are discarded, the only enzyme systems inhibited were choline oxidase, acetylcholine esterase, and pyruvate oxidase.

Cori and his coworkers (24) have studied in detail the effect of methyl-bis-(β-chloroethyl)amine HCl upon the enzymes dealing with phosphorus exchange. They found that phosphocreatine phosphokinase, phosphopyruvate phosphokinase, and inorganic pyrophosphatase were considerably inhibited with 4×10^{-3} M. We found that acid phosphatase was inhibited 40 per cent, while

adenosinetriphosphatase and alkaline phosphatase were not affected with 1×10^{-3} M (Table XI). It could be concluded from these data that none of these enzymes is inhibited at concentrations around the lethal dose. However, Cori found that in certain tissues of animals injected with methyl-bis-(β-chloroethyl)amine HCl, or the ethylenimine transformation product there was partial inhibition of inorganic pyrophosphatase, phosphocreatine phosphokinase, and hexokinase. It may therefore be concluded that these three P exchange enzymes are inhibited by nitrogen mustards at concentrations around the lethal dose.

In Table XII are given data on the effect of TBA and MBA on other enzyme systems. It is surprising to find such highly reacting substances so ineffective on the activity of a large number of enzymes. For example, in spite of the high reactivity of the halogenated alklyamines with —SH groups with which they give compounds of the type succinoxidase, adenosinetriphosphatase,



papain, *d*-amino acid oxidase, phosphoglyceraldehyde dehydrogenase, carboxylase, transaminase—all requiring the presence of —SH groups for activity—were not affected at all by 0.001 M.

The findings that the NH_2 groups of thiamine, nicotinic acid amide, and pyridoxine combine with nitrogen mustards have not been confirmed in enzyme experiments where nitrogen mustard and enzyme were kept at pH 7. The activity of yeast carboxylase which requires diphosphothiamine, of lactate dehydrogenase which requires diphosphopyridine nucleotide, and of transaminase which requires pyridoxal (25) was not affected by nitrogen mustards.

DISCUSSION

The experiments presented in this paper on inhibitions of enzyme reactions by nitrogen mustards show that these compounds act either as structural inhibitors or through combination with different reactive groups of the protein moiety of the enzyme. The halogenated alklyamines in aqueous neutral solutions are rapidly transformed into the quaternary ethylenimonium derivatives which are structurally similar to choline. Inhibition of the oxidation of choline by choline oxidase, of the hydrolysis of acetylcholine by the esterase, and of the synthesis of acetylcholine by choline acetylase, is due in every case to structural inhibition; *i.e.*, inhibition due to combination of the nitrogen mustard derivative with the protein moiety of the enzyme at the same side chains where combination of choline or acetylcholine and protein takes place. Indication that such a specific combination occurs was given by the lack of action of methyl-bis-

(β-chloroethyl)amine on the hydrolysis of monobutyrin, by prevention of inhibition on previous addition of choline or acetylcholine, and partial reversal on addition of choline after nitrogen mustard addition to the enzyme. There is, however, a difference between the other well known structural inhibitors and nitrogen mustards. In fact, while inhibition of succinoxidase by malonate depends on the ratio of malonate: succinate and not on the absolute amount of malonate (see reviews by Wooley (25–27), by Roblin (28), and by Welch (29)), nitrogen mustard inhibition occurs with very small concentrations and is not reversed completely. This must be attributed to strong association between the nitrogen mustard and the protein. This strong association was strikingly demonstrated when different substances known to react easily with nitrogen mustards were used to prevent inhibition. Thiosulfate, the most effective preventive agent, had to be added at a concentration 1000 times as great to prevent inhibition. The nitrogen mustards must, thus, be looked upon as a new subdivision of the general structural inhibitors, effective at low concentrations because of seemingly irreversible combination with the protein at the same specific side chain where combination with choline takes place.

The other enzyme inhibitions studied by Cori, as well as those found by Dixon and by us (inhibition of phosphokinases, pyruvate oxidase, etc.) have a different mechanism of action and require greater concentrations of inhibitor. They must be due to interaction of certain groups of the proteins (NH_2 groups, carboxyl groups, —SH groups) with the highly reactive halogen groups of nitrogen mustard.

SUMMARY

Nitrogen mustards are powerful inhibitors for choline oxidase, acetylcholine esterase, and choline acetylase, half-inhibition of the first enzyme being produced with concentrations around 1×10^{-6} M; *i.e.*, ten times less than the LD_{50} values. Acetylcholine esterase and choline acetylase required higher concentrations. This inhibition seems to be due to the structural similarity of the ethylenimonium derivatives with choline and acetylcholine. A list of enzyme systems inhibited by nitrogen mustards is given.

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THE EFFECT OF NITROGEN MUSTARDS ON ENZYMES AND TISSUE METABOLISM*

II. The Effect on Tissue Metabolism

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It was shown in the preceding paper (1) that nitrogen mustards act as structural inhibitors for the enzymes where choline acts as a substrate, such as choline oxidase, acetylcholine esterase, and choline acetylase. Besides this type of inhibition, nitrogen mustards produced other enzyme inhibitions. *In vitro* studies with enzymes are an indication that such enzymatic reactions might be inhibited in experiments with tissues or in *in vivo* experiments. However, there is no certainty that they would actually be found, not only because the necessary concentrations might not be present, but also because the compound might have been transformed into others of different constitution or might have combined with other substances present in the extracellular milieu. We present in this paper studies on the effect of nitrogen mustards on tissue metabolism. Experiments with tissues permit the study of biochemical reactions which have not yet been accomplished in cell-free systems. These studies have shown that pyruvate metabolism in tissues is powerfully inhibited and that enzyme inhibitions in tissues increase in degree as the time of exposure of nitrogen mustards to tissue increases. Such experiments with tissue slices have finally been followed by a study in tissues of animals receiving nitrogen mustard of the same enzyme reactions that were inhibited in *in vitro* experiments.

Nitrogen Mustards and Tissue Respiration

A study of the effects of nitrogen mustards on the chemical activities of living cells is greatly complicated because of the rapid chemical transformations that these substances undergo when in solution, and by the rate of penetration through the cell membranes.

Toxicological tests have shown that the ethylenimonium derivatives are the most toxic of the transformation products of nitrogen mustards. Their concentration diminishes steadily so that at the end of 24 hours none is left in the case

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of TBA, about 9 per cent in that of MBA, and 47 per cent in that of ethyl-bis-(β-chloroethyl)amine HCl (EBA), as can be seen in Fig. 1 plotted from the data on hydrolysis at 25° given by Golumbic *et al.* (2, 3) and by Fruton and Bergmann (4). In all the experiments with tissue slices, the hydrochloride salts of nitrogen mustards dissolved in Ringer-phosphate buffer (phosphate, 0.02 M) were brought to pH 7.4 and were added to the tissue suspension 15 minutes after solution.

1. Tissue Respiration.— In the experiments with tissue slices and with leucocytes, tissues and cells remained in contact with the nitrogen mustards for

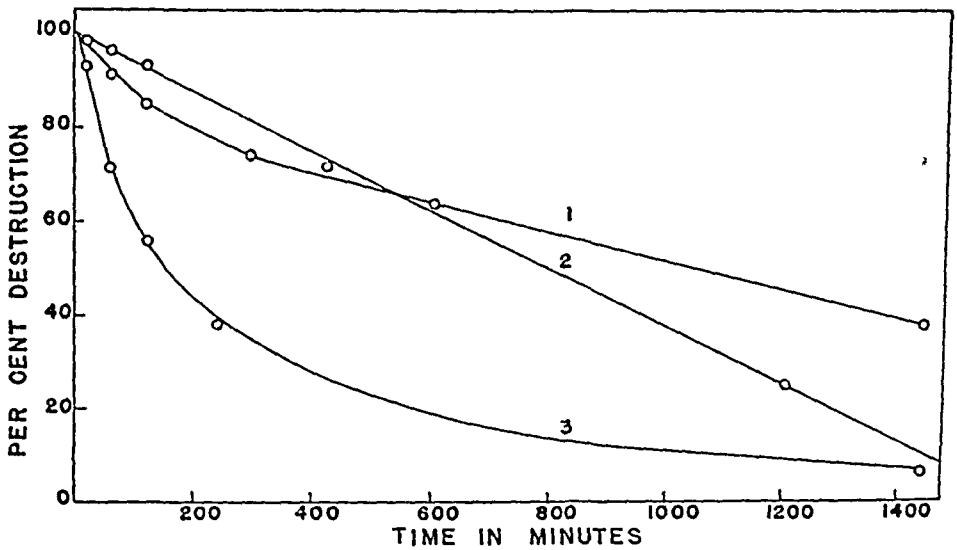

FIG. 1. The destruction of the ethylenimonium transformation product of nitrogen mustards in bicarbonate solution as determined by $Na_2S_2O_3$ consumed in 10 minutes per mM of nitrogen mustard. Temperature 25°. (1) Ethyl-bis(β-chloroethyl)amine; (2) methyl-bis-(β-chloroethyl)amine; (3) tris(β-chloroethyl)amine. (From data given by Golumbic *et al.* (2, 3).)

about 25 minutes before readings of the O_2 uptake were started. There was inhibition of respiration of all tissues studied, and in all cases the inhibition increased gradually so that inhibition in the 2nd hour was always greater than in the first. In some cases, as in that of leucocytes, there was no effect at all in the 1st hour, the inhibition becoming manifest in the 2nd hour (Table I).

The effect of four nitrogen mustards on the respiration of spleen (rat) is given in Table II. All four produced the same progressive inhibition. At the end of 2 hours, ethyl-bis(β-chloroethyl)amine, and isopropyl-bis(β-chloroethyl)amine had the most powerful inhibitory effect.

The respiration of lymphatic tissue (mesenteric and axillary lymph nodes) was inhibited by small concentrations of MBA. In fact, even 1×10^{-5} M (less than the LD_{50} for rabbits) produced definite inhibition (Fig. 2). Marked

TABLE I

Effect of Nitrogen Mustards on Tissue Respirations

Ringer-phosphate buffer. Inhibitor (0.001 M).

Tissue	Inhibition	
	TBA	MBA
	per cent	*per cent*
Leucocytes from myeloid leukemia		
1st hr.		None
2nd "		27.5
Leucocytes from lymphoid leukemia		
1st hr.	8	11
2nd "	13	45
Bone marrow, rat		
1st hr.		21
2nd "		52
Thymus, rabbit		
1st hr.		59
2nd "		88
Lymph nodes, rabbit		
1st hr.	12	15
2nd "	34	70
Liver, rat		
1st hr.	19	30
2nd "	23	72
Kidney, rat		
1st hr.	48	70
2nd "	77	92
Brain, rat		
1st hr.	20	14
2nd "	32	33

TABLE II

Effect of Nitrogen Mustards on the Respiration of Spleen Slices (Rat)

Glucose-Ringer-phosphate, pH, 7.4. Nitrogen mustard, 0.001 M.

Nitrogen mustard	O_2 uptake per mg. dry tissue	
	1st hr.	2nd hr.
	c.mm.	*c.mm.*
None	11.6	10.3
Methyl-bis(β-chloroethyl)amine	10.9	6.1
Tris(β-chloroethyl)amine	11.0	8.6
Isopropyl-bis(β-chloroethyl)amine	7.8	5.4
Ethyl-bis(β-chloroethyl)amine	7.2	4.4

histological alterations and the leucopenia are also indications of this marked sensitivity of lymphoid tissue to nitrogen mustards.

2. Tissue Glycolysis.—Nitrogen mustards inhibit somewhat the activity of hexokinase while they have no effect on the other enzymes which take part in the glycolytic process. In tissue slices, the anaerobic glycolysis of rat brain (slices) was not affected in experiments of 1 hour duration. In rabbit bone marrow there was slight inhibition in the 1st hour which became more marked in the 2nd hour. Anaerobic glycolysis of brain was unaffected.

3. Pyruvate Metabolism.—The metabolism of pyruvate by tissue slices was profoundly affected by nitrogen mustards. To differentiate the effect on

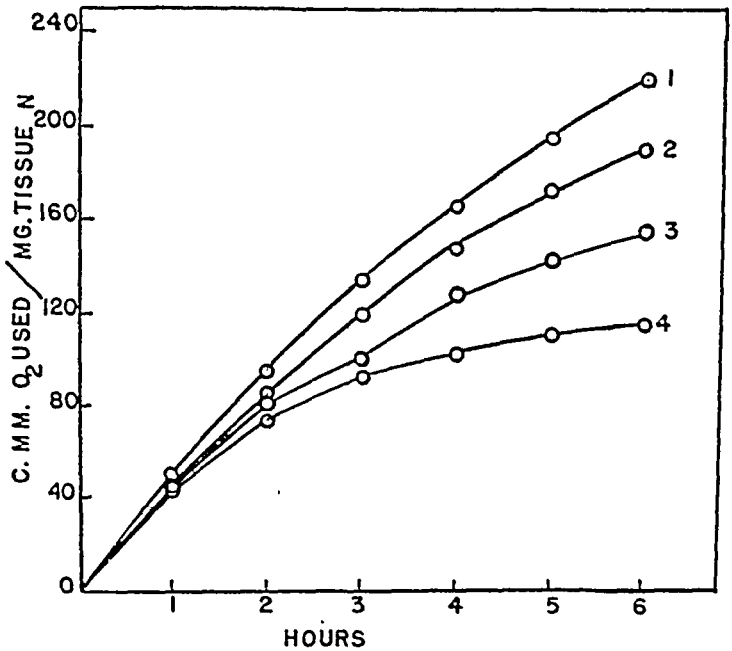

FIG. 2. Effect of various concentrations of MBA on the respiration of rabbit lymphatic tissue. (1) Control; (2) 1×10^{-5} M MBA; (3) 5×10^{-5} M MBA; (4) 1×10^{-4} M MBA.

pyruvate metabolism from the general inhibition of respiration, all the experiments were performed both in the presence and in the absence of pyruvate. The value Q_{O_2} pyruvate $- Q_{O_2}$ would represent the increase in O_2 uptake due to pyruvate oxidation. Furthermore, pyruvate analysis measured the amount of pyruvate utilized by the tissue. Four nitrogen mustards were tested: methyl-bis(β-chloroethyl)amine HCl (MBA), tris(β-chloroethyl)amine HCl (TBA), isopropyl-bis(β-chloroethyl)amine HCl (PBA), and ethyl-bis(β-chloroethyl)-amine HCl (EBA). All of them produced a striking inhibition of pyruvate metabolism at the concentration used (0.001 M), MBA being the most powerful inhibitor (Table III). The same increase of inhibition with time was observed. With 1×10^{-3} M MBA the inhibition started as soon as pyruvate was added to the tissues (30 minutes after addition of nitrogen mustard). With 1×10^{-4} M,

inhibition started only 30 minutes after pyruvate addition and increased with time so that at the end of the last half hour (experiments of 2 hours' duration) the inhibition was 61 per cent. With 5×10^{-5} M, the inhibition started 45 minutes after pyruvate addition. In the last 15 minutes, the inhibition had increased to 55 per cent. Finally with 1×10^{-5} M, the inhibition started only 90 minutes after measurement of the O_2 uptake and 105 minutes after addition of the nitrogen mustard (Fig. 3). The inhibitory effect of nitrogen mustard was also shown on measuring pyruvate utilization (Table IV). It must be mentioned, however, that at the end of 2 hours there is still a large proportion of ethylenimonium compound present in MBA (see Fig. 1). This inhibition of pyruvate metabolism seems to be confined to animal tissues, for the oxidation

TABLE III

Effect of Nitrogen Mustards on the Respiration of Kidney Slices (Rat) and on the Metabolism of Pyruvate

Ringer-phosphate, pH 7.4. Methyl-bis(β-chloroethyl)amine = MBA; tris(β-chloroethyl)-amine = TBA; isopropyl-bis(β-chloroethyl)amine = PBA; ethyl-bis(β-chloroethyl)amine = EBA. The figures give c.mm. per mg. dry weight per hour.

Measurements	Control	MBA	TBA	PBA	EBA
	c.mm.	*c mm.*	*c mm.*	*c mm.*	*c mm.*
O_2 uptake, 1st hr.....	22.0	6.6	9.5	9.2	14.3
" " 2nd " .	17.8	0.8	1.6	4.6	5 0
" " with pyruvate					
1st hr.	32.8	12.4	18.5	17.8	19.8
2nd "	30.5	1.1	6.4	5.9	8.6
Pyruvate utilization, 2 hrs	35.1	6.4	17.3	11.4	12.0

of pyruvate by yeast was inhibited only to a small degree (Fig. 4). As the minimum lethal dose of MBA in subcutaneous injection in mice is 2.6 mg. per kilo, *i.e.* 1.35×10^{-5} M, inhibition of pyruvate metabolism can be obtained with amounts well within the minimum lethal dose.

4. Oxidation of Amino Acids by Kidney Slices.—The effect of nitrogen mustards on the oxidation of amino acids by kidney slices was studied by measuring the O_2 uptake of the tissues in the presence and in the absence of amino acids and by measuring the formation of NH_3. The following amino acids were used: glutamic acid, leucine, valine, and *d,l*-alanine. The oxidation of *l*-amino acids (measured by O_2 uptake as well as by NH_3 formation) was inhibited, that of glutamate showing the greatest effect. The oxidation of *d,l*-alanine (as measured by NH_3 formation) was increased in the presence of four nitrogen mustards (Table V). This increase was investigated further by measuring simultaneously the O_2 uptake, alanine utilization, and pyruvate and NH_3 formation. Both MBA and TBA produced an increase in the utilization of alanine

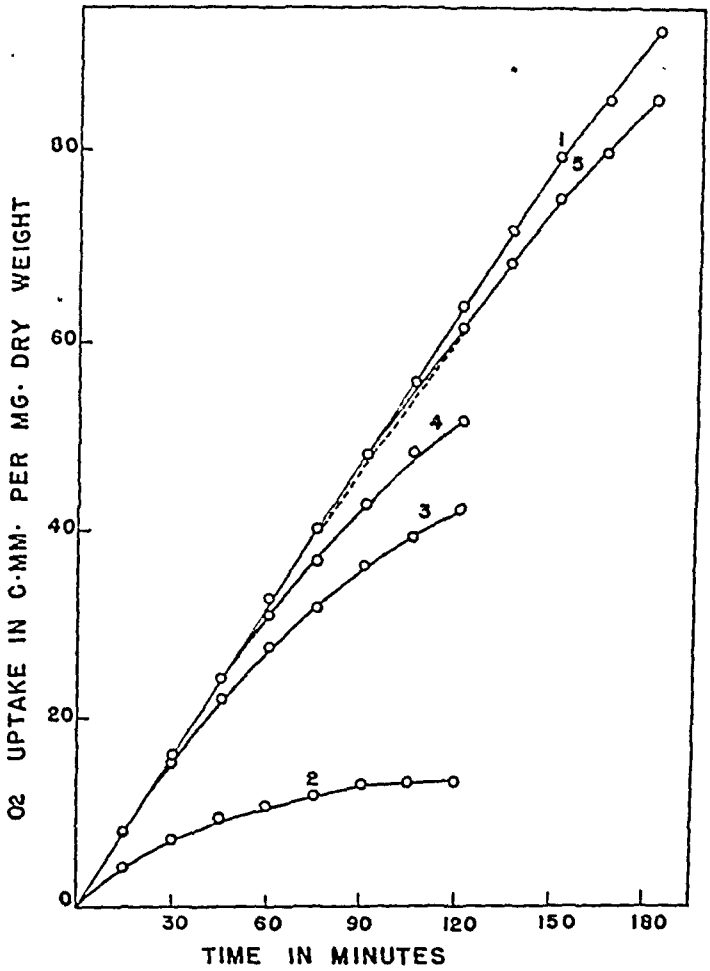

FIG. 3. Effect of methyl-bis(B-chloroethyl)amine (MBA) on the O_2 uptake of kidney slices in the presence of pyruvate. Pyruvate, 0.01 M. pH, 7.4. Temperature 38°. (1) Control; (2) MBA, 0.001 M; (3) MBA, 0.0001 M; (4) MBA, 0.00005 M; (5) MBA, 0.00001 M.

TABLE IV

Effect of Methyl-Bis(β-Chloroethyl)amine (MBA) at Different Concentrations on the Metabolism of Pyruvate by Kidney Slices (Rat)

Figures give pyruvate utilization at the end of 2 hours. Pyruvate utilization, control = 35.1 c.mm.

MBA concentration	Pyruvate utilization MBA	Inhibition
M	c.mm.	per cent
0.001	6.4	82
0.0001	19.8	43.5
0.00005	23.0	34.5
0.00001	30.4	12.4

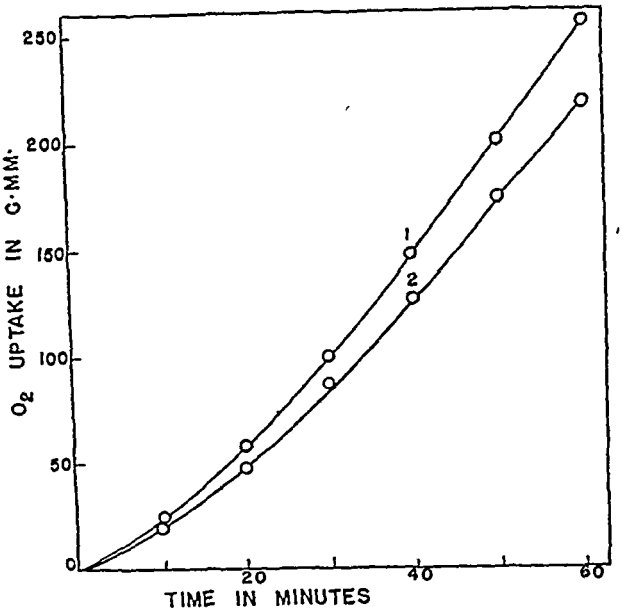

FIG. 4. Effect of methyl-bis(β-chloroethyl)amine HCl (MBA) on the oxidation of pyruvate by baker's yeast. Hippurate buffer, pH 3.8. MBA, 1×10^{-3} M; pyruvate, 1×10^{-2} M. Temperature 28°. (1) Control; (2) MBA, 1×10^{-3} M.

TABLE V

Effect of Nitrogen Mustards on the Oxidation of Amino Acids by Kidney Slices

Ringer-phosphate, pH 7.4; amino acid concentration, 0.01 M; nitrogen mustard, 0.001 M.

Substrate	Nitrogen mustard	O_2 uptake		NH_3 formation	
		Control	Inhibitor	Control	Inhibitor
		c.mm.	*c.mm.*	*c.mm.*	*c.mm.*
Glutamate	TBA	34.3	13.6	0.515	0.26
"	MBA	34.3	11.0	0.515	0.30
Leucine	TBA	22.9	12.6	1.0	0.845
Valine	"	17.9	13.9	0.995	0.885
"	MBA	17.9	11.8	0.995	1.053
Phenylalanine	TBA	12.8	10.0	0.966	0.834
"	MBA	12.8	10.0	0.966	1.011
d, *l*-Alanine	TBA	35.3	32.2	0.644	1.97
"	MBA	35.3	36.0		2.70
"	PBA	45.3	27.5	2.04	2.65
"	EBA	42.5	23.6	1.95	2.53

accompanied by an increased formation of pyruvate and NH_3. In the control experiments, 94 per cent of the pyruvate formed through the oxidation of

alanine was further utilized by the tissues while 83 per cent of the NH_3 formed was found in the solution. In the presence of MBA, 66 per cent of the pyru vate formed was found, while the accumulation of NH_3 was the same as in the control (Table VI).

TABLE VI

Effect of Nitrogen Mustards on the Oxidation of d,l-Alanine

Incubation time, 90 minutes. Ringer-phosphate, pH 7.4. Alanine concentration, 0.04 M. Tissue, rat kidney slices. Inhibitor, 1×10^{-3} M. Figures give micromoles per milligram of dry tissue.

	Control	TBA	Inhibition or increase	MBA	Inhibition or increase
	μM per mg.	*μM per mg.*	*per cent*	*μM per mg.*	*per cent*
O_2 uptake	1.66	1.34	−19	1.04	−31
Alanine utilization	1.11	1.62	+46	1.74	+57
Pyruvate formation	0.06	0.82	+1200	1.15	+1816
NH_3 "	0.92	1.25	+36	1.44	+46

TABLE VII

Effect of Nitrogen Mustards on Biochemical Reactions Leading to Synthesis

Synthesis	Tissue	TBA	Inhibition	MBA	Inhibition
		M	*per cent*	*M*	*per cent*
1. Carbohydrate, from pyruvate	Kidney	10^{-3}	71	10^{-3}	94
" " "	"	10^{-4}	61	10^{-4}	71
2. Urea, from glucose + NH_3	Liver	10^{-3}	68	10^{-3}	89
" " " " "	"	5×10^{-4}	62	5×10^{-4}	75
" " " " "	"	1×10^{-4}	2	1×10^{-4}	2
3. Creatine, from glycocyamine + methionine (3 hrs.)	"	1×10^{-3}	28	1×10^{-3}	26
Creatine, from glycocyamine + methionine (6 hrs.)	"	"	62	"	79
4. Amino acid, from NH_3 + ketoglutarate	Kidney	"	32	"	40

5. Effect of Nitrogen Mustards on Biochemical Reactions Leading to Synthesis.—On observing animals that have received toxic amounts of halogenated alkylamines, one is struck by the slow effect of these substances, which never produce rapid death; by the progressive disintegration of the animals. The partial inhibition of oxidation reactions accompanied by inhibition of those reactions leading to synthesis and to regeneration of tissue cells might be one of the mechanisms of the toxic action of halogenated alkylamines. The experiments reported in Table VII show that methyl-bis(β-chloroethyl)amine and tris(β-chloroethyl)amine inhibit those reactions leading to synthesis that have

been studied. The synthesis of carbohydrate from pyruvate by kidney slices was inhibited by TBA and by MBA. The synthesis of creatine by liver slices in the presence of glycocyamine and methionine was inhibited with both halogenated alkylamines, TBA and MBA. The synthesis of glutamic acid by the kidney in the presence of α-ketoglutarate plus NH_4Cl was inhibited by TBA and MBA. This marked effect of halogenated alkylamines on enzymatic processes leading to synthesis might explain the delay in the appearance of leucopenia. The compounds seem to act by slowing multiplication processes. This was beautifully shown by Friedenwald and Schultz (5), who found that amounts of alkylamine too small to inhibit the metabolism of the cornea produce inhibition of mitosis, and by the experiments of Auerbach *et al.* (6), who found that sublethal doses of MBA reduced the fertility of female *Drosophila* and produced considerable effects on mutation rate and chromosome arrangement as well as disturbances in the nuclear mechanism. Whether these effects are due to the halogen groups and are thus similar to the effects produced by mustard gas, or are due to structural inhibition of enzymatic reactions where choline is one of the substrates is not yet known. It is quite possible that some of these toxic symptoms may be due to disturbances of choline metabolism.

6. Bone Marrow.—It is known that nitrogen mustards produce marked alterations in the bone marrow even when used at concentrations far below the lethal dose. A series of experiments was performed with rabbit bone marrow slices with the purpose of finding an agent which would either prevent or reverse the injurious effects of nitrogen mustards when used for medical treatment. To insure a steady respiration, the slices were suspended in the following solution, which was shown by Warren (7) and by Goldinger *et al.* (8) to maintain respiration: 30 cc. of neutralized, sterile, unheated, beef serum ultrafiltrate, 30 cc. of Ringer solution (100 cc. of 0.154 M NaCl, 2 cc. 0.154 M KCl, 2 cc. 0.11 M $CaCl_2$), 40 cc. of 0.1 M phosphate, pH 7.4. Glucose was added to a concentration of 0.01 M. MBA at a concentration of 1×10^{-3} M inhibited the respiration 50 per cent in the 1st hour and 65 per cent in the 2nd hour. With low concentrations, the effect was similar to that produced in kidney slices. 5×10^{-5} M MBA, which had no effect on the respiration in the 1st hour, produced 30 per cent inhibition at the end of 5 hours. Even a concentration of 5×10^{-6} M gave a definite though small inhibition (Fig. 5). (This amount is about half the initial concentration in the blood of patients treated with MBA.)

With low concentrations of MBA most of it became fixed in the tissue and very little remained in the solution. This was demonstrated by keeping the slices in MBA (5×10^{-5} M) for 30 minutes at 38°. The slices were then washed and put into Warburg vessels containing no nitrogen mustard. Other slices were suspended in the fluid in which the previous slices had been kept, which originally contained 5×10^{-5} M MBA. At the end of 5 hours the respiration of the washed slices was inhibited 26 per cent, while that of the slices suspended in the fluid of the previous slices was inhibited only 14 per cent (Fig. 6).

Some preliminary experiments performed in 1943 indicated that choline may protect mice against lethal doses of MBA. The experiments in Fig. 7 show that choline added to bone marrow slices protected the tissue against the inhibition of respiration by MBA. Since choline in large concentrations inhibits tissue respiration, experiments were performed to find the maximum amount of choline which may be added to tissues without affecting respiration. Tissue slices were kept suspended for 45 minutes in the buffered solution containing

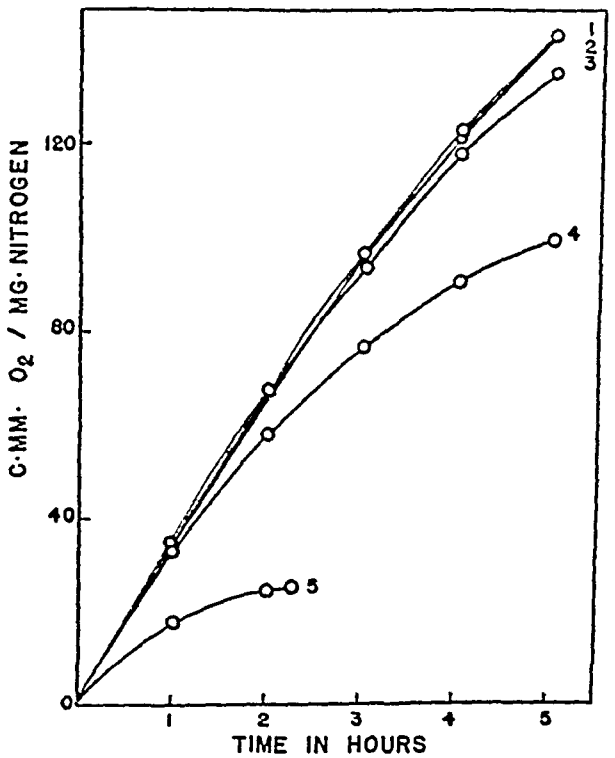

FIG. 5. Inhibition of bone marrow respiration by nitrogen mustards. (1) Control; (2) MBA, 1×10^{-6} M; (3) MBA, 5×10^{-6} M; (4) MBA, 5×10^{-5} M; (5) MBA, 1×10^{-3} M.

5×10^{-3} M choline before addition of nitrogen mustard (5×10^{-5} M). Tissues thus treated were scarcely affected by MBA, while in the absence of choline, MBA inhibited respiration by 43 per cent. A number of substances which enter into the cycle of biological choline interrelationships were tried as preventive agents: dimethylaminoethanol, betaine, methionine, and lecithin. Lecithin and betaine were without protective effect. 3×10^{-3} M dimethylaminoethanol plus 3×10^{-3} M methionine prevented almost completely (93 per cent) the inhibitory action of 5×10^{-5} M MBA;[1] dimethylaminoethanol alone

[1] Dimethylaminoethanol, at a ratio of 100:1 of nitrogen mustard, prevented the inhibition of choline oxidase by 53 per cent.

prevented it 81 per cent, while methionine alone had no effect at all (Fig. 8). Although the mechanism of this protective action has not yet been elucidated, it undoubtedly is an indication of the important rôle of choline in the metabolism of bone marrow. The methylation of dimethylaminoethanol to choline was demonstrated by du Vigneaud *et al.* (9). These experiments suggest the

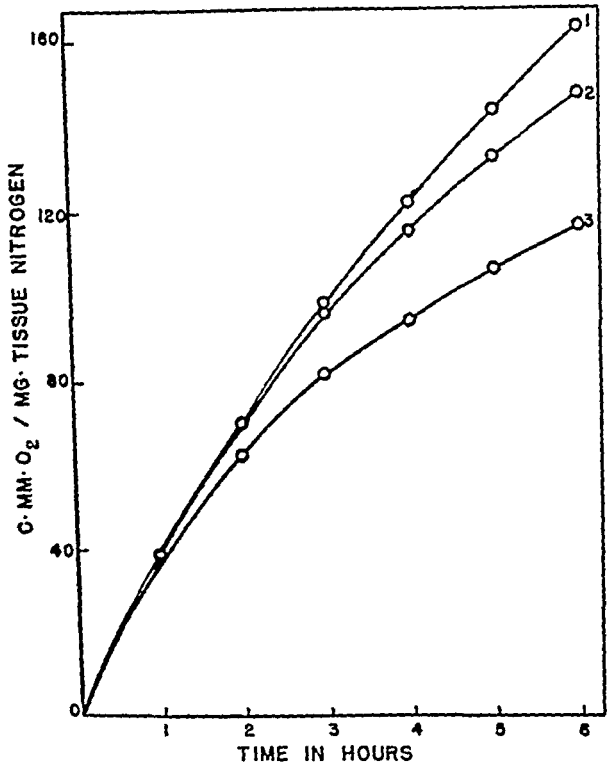

FIG. 6. Fixation of nitrogen mustards by bone marrow. (1) Control; (2) fresh bone marrow slices added to centrifuged supernatant fluid obtained from 30 minutes' incubation of other bone marrow slices (No. 3 below) with MBA, 5×10^{-6} M; (3) washed bone marrow slices from the above 30 minutes' incubation with MBA were resuspended in fluid free of MBA.

utilization of choline or choline precursors to avoid the injurious effects of nitrogen mustards on the bone marrow during nitrogen mustard therapy.

7. Toxicity of Methyl-Bis(β-Chloroethyl)amine to Growing Seeds.—Water solutions of nitrogen mustards retain toxicity for a longer time than nitrogen mustards in well buffered solutions. To study the toxicity of these water solutions to plants, tomato seedlings were grown in sand and distilled water in the presence and in the absence of 0.001 M methyl-bis(β-chloroethyl)amine. For 3 days the seedlings in both grew about equally. At the end of the 3rd day the

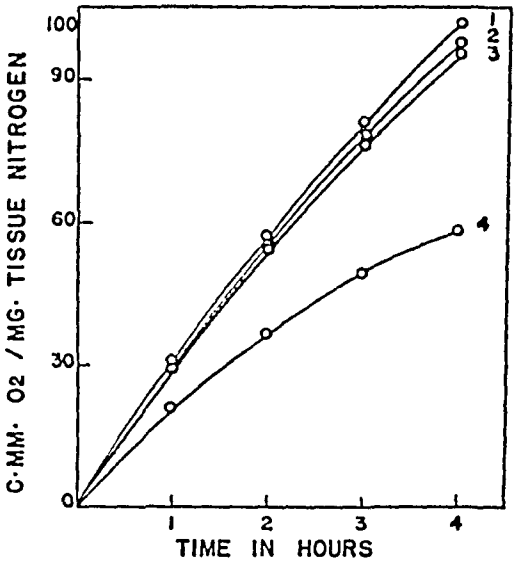

FIG. 7. Protection of nitrogen mustard inhibition of respiration by choline. (1) Control, bone marrow slices; (2) bone marrow + 5×10^{-3} M choline; (3) bone marrow kept for 45 minutes with 5×10^{-3} M choline; 5×10^{-5} M MBA, added afterwards; (4) bone marrow + 5×10^{-5} M MBA. O_2 uptake measurements are those obtained after the 2nd hour of incubation.

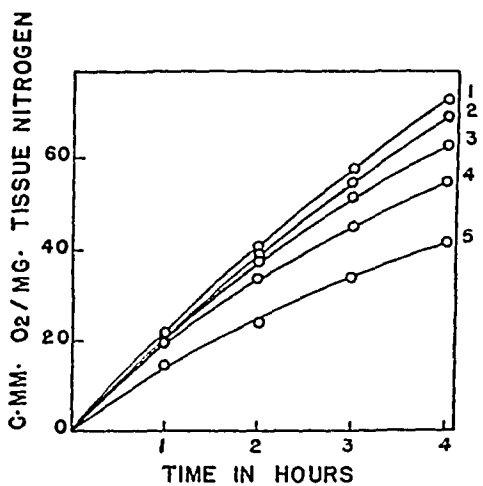

FIG. 8. Protection of nitrogen mustard inhibition of respiration by dimethylaminoethanol. (1) Bone marrow control; (2) dimethylaminoethanol, 3×10^{-3} M; (3) dimethylaminoethanol, 3×10^{-3} M + 3×10^{-3} M methionine + 5×10^{-5} M MBA; (4) dimethylaminoethanol, 3×10^{-3} M + MBA, 5×10^{-5} M; (5) MBA, 5×10^{-5} M. O_2 uptake measurements are those obtained after the 2nd hour of incubation.

seedlings containing MBA ceased to grow. At the end of 1 week the difference between the control seeds and the seeds containing MBA was remarkable.

Nitrogen mustards in water are thus toxic to growing seeds and the toxicity is manifested days after treatment.

Tissue Metabolism and the Activity of Some Enzyme Systems in Rats Treated with Methyl-Bis(β-Chloroethyl)amine (MBA)

In studies with isolated enzyme systems it has been shown that the most striking property of the halogenated alkylamines is the inhibition of choline oxidase, and next to it the inhibition of choline esterase, pyruvate oxidase, phosphocreatine phosphokinase, and inorganic pyrophosphatase. In studies with tissue slices it was found that a number of reactions leading to synthesis were inhibited as well as the total respiration. Whether these findings can be extended to the effect of these compounds on the living animal will depend on the distribution throughout the different tissues of this or that transformation product of the halogenated alkylamines. In searching for the mechanism of the toxic action of a compound, studies on enzymes must therefore be accompanied not only by observations of the enzymatic reactions in tissues but also by a determination of the enzyme activity of the tissues of animals treated with lethal amounts of alkylamine. Inhibitions found under these conditions may reasonably be considered among the factors responsible for toxicity. For this purpose rats were treated with amounts of MBA which allowed a survival up to 100 hours. Twelve rats together in each experiment received methyl-bis(β-chloroethyl)amine in a gas chamber and the rats were killed at different times for study of tissue metabolism.

1. Choline Oxidase by the Liver and Kidney of Rats Treated with MBA.—Kidney slices of rats treated with MBA and killed 2 and 5 hours after treatment showed a decreased Q_{O_2} value in the absence of choline (27 per cent decrease) when compared to the Q_{O_2} value of normal rats. On addition of choline there was no rise of the Q_{O_2} values 2 hours after treatment; *i.e.*, there was complete inhibition of choline oxidase. Complete inhibition was also found in rats killed 19 hours and 43 hours after treatment. The inhibition of choline oxidase was partial in the other rats and had disappeared at the end of 48 hours, an indication that in 48 hours there was new formation of the enzyme (Table VIII) (the inhibition produced by the alkylamines is irreversible). This resynthesis of the enzyme in such a short time is remarkable and is indicative that the continuous breakdown and synthesis of protein in the body apply also to the protein moiety of enzymes. The inhibition of choline oxidase in the kidney was confirmed by Cori (10).

In sharp contrast with this inhibition of choline oxidase in the kidney was the lack of effect in the liver. The choline oxidase activity of the treated rats, killed from 1 hour after treatment to 30 hours after, remained unimpaired.

2. Pyruvate Oxidation by the Kidney.—It has been shown that the oxidation of pyruvate by kidney slices is strongly inhibited by nitrogen mustards. The

TABLE VIII

Choline Oxidation by the Kidney of Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine (MBA)

Q_{O_2} of normal kidney in the absence of choline = 18.3. In the presence of choline = 27.0: Q_{O_2} choline 8.7.

Amount of MBA	Time after gassing	Q_{O_2} without choline	Q_{O_2} with choline	Q_{O_2} choline	Inhibition of choline oxidation
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*c.mm.*	*c.mm.*	*per cent*
0.71	2	13.0	13	0	Complete
0.71	5.2	13.5	18.2	4.2	51.7
0.71	12	18.5	21.6	3.1	64.5
0.80	19	16.0	14.3	0	Complete
0.80	24	10.0	16.8	6.8	21.8
0.80	31	18.5	19.3	0.8	90.8
0.80	43*	19.4	18.0	0	Complete
0.80	46.3	20.4	23.9	3.5	59.7
0.65	48	11.4	22.5	11.1	None

* Rat died 40 minutes before experiment started.

TABLE IX

Pyruvate Oxidation by the Kidney of Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine (MBA)

Q_{O_2} of normal kidney in the absence of pyruvate = 18.3. In the presence of pyruvate = 27.5: Q_{O_2} pyruvate = 9.2.

Amount of MBA	Time after gassing	Q_{O_2} without pyruvate	Q_{O_2} with pyruvate	Inhibition of pyruvate oxidation
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*c.mm.*	*per cent*
0.71	2	13.0	11.4	Complete
0.71	5.2	13.6	13.9	Complete
0.71	12	18.5	20.3	80.5
0.80	19	16.0	13.2	Complete
0.80	24	10.0	14.9	46.7
0.80	31	18.5	27.3	None
0.80	43	19.4	23.9	51.0
0.80	46.3	20.4	25.0	50.0
0.65	48	11.4	12.7	86.0
0.65	72	24.2	23.3	Complete
0.46	83	18.4	21.7	35.8

same inhibition was found in the kidney of rats treated with MBA. The inhibition was complete in rats killed 2, 5.2, 19, and 72 hours after treatment. In all the other rats, killed at different times up to 83 hours, there was partial inhibition (Table IX).

Whether the inhibition of pyruvate oxidation is found in all tissues or is confined to the kidney is not known. In one case, the oxidation of pyruvate by the

brain of a rat killed 2 hours after gassing was found to be the same as in normal rats. In another case, the utilization of pyruvate by the liver of a rat killed 24 hours after treatment was also found to be normal.

3. Urea Synthesis by the Liver.—It has been shown that the synthesis of urea by the liver is inhibited by alkylamines. In rats treated with MBA the synthesis of urea by the liver was followed, the animals being killed at different times after treatment, from 1 hour to 74 hours. In two rats which died half an hour before the experiments started there was 83 and 78 per cent inhibition of urea

TABLE X

The Activity of Urea Synthesis by the Liver in Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine

The average value of Q urea of normal rats is: 4.87 (cubic millimeters of urea produced per milligram of tissue (liver) per 2 hours).

Amount of MBA	Time after gassing	Q urea	Inhibition
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*per cent*
0.7	1	3.49	28.3
0.7	4.7	4.37	10.0
0.7	10.5	4.03	9.6
0.7	21	5.89	None
0.7	24	4.21	13.5
0.71	26	3.86	20.7
0.71	31	1.85	62.0
0.71	29	3.72	23.6
0.71	43*	0.81	83.2
0.71	74	3.72	23.6
0.71	46.5*	1.04	78.5
0.71	53	3.72	23.6
0.71	50	2.60	46.5

* Rat died ½ hour before the experiment started.

synthesis. In all the other cases, except one, there was partial inhibition (Table X).

4. Brain Choline Esterase.—It has been shown by Dixon, Thompson, and ourselves that brain choline esterase is inhibited by nitrogen mustards. However, the amount required to produce about 60 per cent inhibition was 1×10^{-4} M, which is 100 times higher than the lethal dose. As expected from these figures the choline esterase activity of the brain of rats killed from 1 hour to 55 hours after gassing remained remarkably within normal values.

5. Tissue Glycolysis and Coenzymes.—In *in vitro* experiments halogenated alkylamines produced some inhibition of glycolysis 1½ hours after the addition of alkylamine to the tissue. The anaerobic glycolysis of brain slices of rats treated with methyl-bis(β-chloroethyl)amine remained within normal values at different times after treatment.

The diphosphopyridine nucleotide and diphosphothiamine content of tissues was also normal, showing that MBA had no effect at all on these coenzymes.

Cori (10) injected methyl-bis(β-choroethyl)amine HCl (15 mg. per kilo) into rats. At the end of 3 hours, the following enzymes from the kidney were inhibited: choline oxidase, 100 per cent; hexokinase, 74 per cent; inorganic pyrophosphatase, 42 per cent. When the renal blood vessels were clamped for 20 minutes after the injection of nitrogen mustard choline oxidase inhibition dropped to 32 per cent. It seems that all the toxic substances capable of inactivating enzymes disappear from the circulating blood stream 20 minutes after injection.

DISCUSSION

In the experiments presented in this paper nitrogen mustards added to tissue slices produced a strong inhibition of pyruvate metabolism. They also inhibited the oxidation of *l*-amino acids and the utilization of NH_3. Furthermore there was inhibition of a number of synthesis reactions, such as the synthesis of carbohydrate from pyruvate, the synthesis of creatine from glycocyamine and methionine, the synthesis of glutamic acid, and the synthesis of urea. In plants, there was inhibition of the growth of seeds. Of these inhibitions, the inhibition of methylation reactions, such as the transfer of the methyl group from methionine to glycocyamine with the formation of creatine, perhaps belongs to the type of structural inhibition because labile methyl groups are found only in quaternary nitrogen compounds structurally similar to the active ethylenimonium derivative of nitrogen mustards. The inhibition of pyruvate metabolism and of *l*-amino acid oxidation might be due either to combination with the -SH groups of the protein moiety of these enzymes or to combination of other groups of the side chains of the protein with the highly reactive halogen groups of nitrogen mustards. The lack of inhibition of succinoxidase and phosphoglyceraldehyde dehydrogenase (which are sulfhydryl enzymes) is only evidence that nitrogen mustards do not inhibit all sulfhydryl enzymes. In fact, inhibition of mitosis by nitrogen mustards, sulfur mustard, and x-rays finds reasonable explanation in the assumption that the process is controlled by a sulfhydryl enzyme with very labile and reactive -SH groups, easily alkylated by the first two and oxidized by the last.

The great sensitivity of the bone marrow respiration explains the leucotoxic action of nitrogen mustards. The experiments on bone marrow slices in which the inhibitory action of nitrogen mustards was prevented on addition of choline or the choline precursors, dimethylaminoethanol and methionine, seem to demonstrate the rôle of choline in the metabolism of the bone marrow, and they point the way for the treatment of nitrogen mustard intoxication in the course of its medical use.

The mechanism of the toxic action of nitrogen mustards is thus complex and

must be due to the dual action of the compound which can act as a structural inhibitor and an inhibitor of enzyme reactions through combination with certain groups of the protein moiety essential for enzyme activity.

The inhibition of reactions leading to synthesis, the inhibition of pyruvate metabolism, of *l*-amino acid oxidation, of phosphokinases and of hexokinase together contribute to the striking symptoms of destruction and lack of formation of new cells leading to leucopenia and atrophy of the bone marrow. This inhibition of growth was clearly demonstrated in the inhibition of the growth of seedlings treated with nitrogen mustards. Furthermore, the large accumulation of NH_3, in the tissues where amino acids and nitrogen mustards were present, is indication of lack of utilization of NH_3 for the formation of nitrogenous compounds among which the proteins are obviously included.

SUMMARY

Nitrogen mustards at a concentration forty times the minimum lethal dose inhibited the respiration of all tissues studied but affected anaerobic glycolysis very little. The inhibiting effect increased with time. The respiration of lymphoid tissue was extremely sensitive to nitrogen mustard, as concentrations below the LD_{50} definitely inhibited the respiration of rabbit lymph nodes. In tissue slices nitrogen mustards inhibited the oxidation of pyruvate and of *l*-amino acids and the utilization of NH_3. A number of synthesis reactions were also inhibited, such as the synthesis of carbohydrate, of creatine, and of urea. When added to growing seeds, nitrogen mustards inhibited their growth. In rats given lethal doses of nitrogen mustards there were found complete inhibition of choline oxidation and strong inhibition of pyruvate oxidation by the kidney and partial inhibition of urea synthesis by the liver. Inhibition of bone marrow respiration by nitrogen mustards was prevented by the addition of choline, and of dimethylaminoethanol plus methionine The possible mechanism of nitrogen mustard intoxication is discussed.

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VARIATION OCCURRING IN GROUP A STREPTOCOCCI DURING HUMAN INFECTION

Progressive Loss of M Substance Correlated with Increasing Susceptibility to Bacteriostasis

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During the course of routine typing of group A streptococci recovered from nasopharyngeal cultures of patients suffering from hemolytic streptococcal infections of the upper respiratory tract, it was noted that, although there was no change in the colony morphology on sheep or rabbit blood agar plates, the streptococci obtained during convalescence often produced less type-specific M substance than the streptococci isolated during the acute phase of infection. It was also observed that the streptococci isolated in the convalescent period were often susceptible to the bacteriostatic action of normal children's blood, whereas the microorganisms isolated in the early period of the infection were invariably resistant.

These findings suggested that following infection in man the antigenic composition of the hemolytic streptococcus may undergo changes similar to those known to occur in the pneumococcus (1) and diphtheria bacillus (2) following natural infections with these bacteria.

Todd (3) and more recently Ward and Lyons (4) have shown that the ability of hemolytic streptococci to multiply in normal human blood is a reliable and sensitive method for distinguishing different strain variants and for determining mouse virulence. Hare (5) has also reported that saprophytic strains of hemolytic streptococci isolated from the human birth canal can be differentiated from pathogenic strains by their susceptibility to the bactericidal action of human blood. Furthermore, various workers (6–9) have shown that the type-specific M antigen is necessary for the exhibition of virulence of group A streptococci; and that the presence of this protein antigen characterizes the matt variant, whereas the glossy variant produces little or no M substance.

Since previous reports of hemolytic streptococcal variation have been confined for the most part to *in vitro* or animal studies, a similar study was undertaken to investigate this phenomenon during the natural course of infection in man. For this purpose the cultures were obtained at weekly intervals from patients during the acute, convalescent, and carrier stages of their streptococcal respiratory infections; and the individual strains were subjected to tests for their ability to resist the bacteriostatic action of normal human blood and to synthesize the type-specific M protein antigen.

Experimental Methods

The source of the hemolytic streptococcal strains, methods, and preparation of material employed was as follows:—

Hemolytic Streptococci.—The streptococcal strains were recovered from nose and throat cultures of patients with acute hemolytic streptococcal infections of the upper respiratory tract at the time of hospital admission and at weekly intervals thereafter during their hospital stay. In some instances streptococci were not found every week during the convalescent period. A number of patients received therapeutic doses of sulfadiazine for 7 to 14 days between the 2nd and 4th weeks of illness in an effort to clear up their carrier state; several were also treated in a similar manner for purulent complications. Since this therapy often failed to clear the carrier state, subsequent strains were usually obtained from these patients.

The culture swabs were streaked immediately in duplicate on fresh 5 per cent rabbit and sheep blood agar plates and incubated at 37°C. for 18 to 24 hours. Several representative colonies of all hemolytic streptococci were picked and streaked on fresh blood agar plates for further identification or transferred directly to broth for serological grouping and typing by the precipitin method (10, 11). Following isolation and classification, the hemolytic streptococci were immediately lyophilized.

To provide uniform handling and to maintain as nearly as possible the original conditions of the culture, each strain was taken from the lyophilized stock and subcultured in 45 cc. of Todd-Hewitt broth. Following incubation at 37°C. for 16 to 18 hours, the streptococci were collected by centrifugation, resuspended in 2 cc. of fresh broth, and distributed in 0.2 cc. portions into a series of small glass tubes which were kept in the dry CO_2 ice box until used.

Bacteriostasis of Group A Streptococci in Normal Human Blood.—The details of the method to determine the capacity of hemolytic streptococci to resist phagocytosis and multiply in normal human blood have been described (12). In this study no added antibody was employed in the bacteriostatic experiments. The day before each experiment the tubes containing the series of strains obtained from a single patient were thawed from the frozen state, a loopful from each was inoculated into 45 cc. of Todd-Hewitt broth, and incubated for 10 to 12 hours in a water bath at 37°C. The streptococcal cells were separated by centrifugation and resuspended in the original volume of fresh broth. Tenfold serial dilutions varying from 10^{-1} through 10^{-6} were prepared from 1 cc. of each culture for use in the bacteriostatic tests; the remainder of the culture was used to prepare the M extracts.

Titration of Type-Specific M Protein Antigen.—Crude extracts made by heating streptococci with hydrochloric acid were prepared as described by Lancefield (10) from each culture studied after removal of a sample for use in the bacteriostatic tests described above. Twofold serial dilutions of these extracts in saline were tested with constant amounts of homologous type-specific absorbed rabbit antiserum in capillary pipettes as described by Swift, Wilson, and Lancefield (11). The titre of the type-specific M protein antigen was taken as the highest dilution of the extract which gave a positive precipitin reaction with the homologous rabbit antiserum. The group-specific C-anti-C reaction was not involved in these titrations, as antibody to the C carbohydrate had been completely removed from the sera by absorption with heterologous-type group A streptococcal cells.

EXPERIMENTAL RESULTS

Resistance of Strains to the Bacteriostatic Action of Normal Human Blood.—Two hundred and fifty-one strains isolated from 54 patients, who suffered 56 different streptococcal infections, were tested for their ability to resist the

bacteriostatic action of normal human blood. The period of observation for each patient ranged from 4 to 55 weeks (average 11 weeks) during which time cultures were made at weekly intervals. From 2 to 15 strains (average 4.5) of the same serological type were obtained on culture from each patient following his infection. Although cultures taken during the 1st week of infection were invariably positive for group A streptococci, those taken later in convalescence were often negative; and in the case of some patients, many weeks separated cultures positive for these microorganisms. In some instances it is not possible, therefore, to state the precise time at which the strains began to lose resistance to the bacteriostatic action of normal blood.

The strains isolated on the initial culture taken within the 1st week of the patients' infections always resisted bacteriostasis and multiplied in all dilutions of the culture from 10^{-1} through 10^{-6}. Such strains are arbitrarily termed "highly resistant." Strains termed "resistant" signify at least a ++ diminution in growth in the 10^{-6} dilution as compared with the corresponding dilution of the initial culture; those strains termed "susceptible" and "highly susceptible" signify similar diminutions in growth in the 10^{-5} and 10^{-4} dilutions respectively. Results with this test have been highly reproducible; and we believe a ++ growth difference in a single dilution is significant. Some of the "highly susceptible" strains showed much greater differences and failed to grow in culture dilutions as low as 10^{-2}, which is equivalent to the destruction of 100,000 streptococcal cells by 0.25 cc. of normal human blood.

Resistant strains of streptococci were found throughout the entire period of observation among 33, or 59 per cent of the 56 infections studied. In the first part of Table I are recorded the serological types of these resistant strains and the last week after infection that each was isolated, which also corresponds to the period of observation. Similar data with regard to the strains which lost resistance to bacteriostasis are tabulated in Table II. From these data it is apparent that in 23, or 41 per cent, of the infections the strains showed a progressive decrease in resistance. Thus, although the streptococci in the initial cultures were always "highly resistant," strains from 13 infections changed during the period that the patient was studied to "highly susceptible" to the bacteriostatic action of normal human blood; an additional 8 became "susceptible;" and 2 others became "resistant." It is also apparent that the time of development of this change was variable despite the fact that the exact time the change occurred could not always be ascertained. The change from "highly resistant" to "resistant" occurred as early as the 2nd week but was frequently noted in the 7th or 8th weeks; the change to "susceptible" took place as early as the 2nd week and as late as the 19th week; and in half the cases studied in which the strains changed to "highly susceptible," this occurred before the 8th week, while in the remainder this degree of change was delayed up to 24 weeks. Those strains which showed decreased resistance were not necessarily carried

by their hosts longer than the strains which remained fully resistant to bacteriostasis.

A comparison of the length of time that patients with persistently resistant strains were studied with the time at which susceptibility first became apparent in the susceptible strains shows that the former were observed long enough to

TABLE I

*Duration of Resistance to Bacteriostasis of Group A Streptococci Isolated from Patients at Weekly Intervals Following Infection**

Serological type	Strains maintaining resistance‡		Strains losing resistance§	
	No. of infections	Observation period of patients with resistant strains: last wk. in which streptococci were isolated	No. of infections	1st wk. in which susceptible variants appeared
1	1	5	2	2, 3
3	3	5, 11, 13	2	3, 16
5	1	7		
6	2	10, 26	3	3, 5, 6
12	2	4, 4		
14	1	7	1	8
17			1	8
18	1	5		
19	14	5, 5, 5, 6, 6, 6, 7, 7, 8, 11, 12, 12, 19, 21	7	3, 6, 6, 7, 9, 9, 10
26			3	7, 19, 21
29			1	4
30	3	4, 7, 8		
32	1	20		
33			1	2
38	1	6	1	3
NC‖	3	5, 18, 20	1	2

* Streptococci not obtained on every culture from each patient.

‡ From 33 patients (33 infections) 126 strains isolated at weekly intervals maintained resistance to bacteriostasis throughout observation period.

§ From 21 patients (23 infections) 125 strains were isolated at weekly intervals: Susceptible variants first appeared at the times indicated.

‖ NC indicates strains not classified with available sera.

detect such strain variations if they had occurred (Table I). Some of the resistant strains were carried in the patient's throat as long as 20 to 26 weeks following the onset of infection, and most of the patients were under observation for longer periods than it took for variants to appear in the series in which the streptococci became susceptible. This comparison is shown in parallel columns in Table I. The majority of the strains which showed lowered resistance developed these variants comparatively early: 13 within the first 6 weeks, 9 others within 10 weeks, and the remaining 3 within 16, 19, and 21 weeks respectively.

TABLE II

Strains of Group A Streptococci Losing Resistance to Bacteriostatic Action of Normal Children's Blood

Serological type	Bacteriostasis of 125 streptococcal strains isolated at weekly intervals from 21 patients following 23 different infections*			
	Highly resistant‡	Resistant‡	Susceptible‡	Highly susceptible‡
	Wk. following infection streptococci were isolated			
1	1, 2		3, 4	6, 7, 8, 9, 10, 11, 12
1	1	2	3, 4, 5	
3	1			16
3	1, 2	3		4, 5
6	1, 2, 4		6, 10, 16	20
6	1		3, 4, 5, 6, 8, 10, 14	
6	1, 2, 3, 4		5, 6, 7, 9	
14	1, 2, 3, 4, 5, 6, 7	8	9, 10	11
17	1, 2, 3, 4, 5, 6, 7	8, 15	17, 20	24, 28, 51, 55
19	1		9	
19	1		10	
19	1	7		
19	1		9	
19	1			6, 7
19	1	3, 6		
19	1, 5			6
26	1, 2, 3			21
26	1, 2, 6, 15		19, 23	
26	1, 2, 3, 4, 5, 6		7	8, 14, 19
29	1, 2, 3			4
33	1			2, 4, 8
38	1		3, 5, 7	
NC§	1		2, 3	11

* Streptococci not obtained on every culture from each patient.

‡ "Highly resistant" signifies resistance to bacteriostasis in all dilutions of culture from 10^{-1} through 10^{-6}; "resistant" signifies at least a ++ diminution in growth in the 10^{-6} dilution as compared with the corresponding dilution of the initial culture; "susceptible" and "highly susceptible" signify similar diminutions in growth in the 10^{-5} and 10^{-4} dilutions respectively.

§ NC indicates strains not classified with available sera.

Variants were recovered from 9 of 25 patients who received therapeutic doses of sulfadiazine for 7 to 14 days in an effort to clear up the carrier state or purulent complications. From the remaining 16 patients treated with sulfadiazine, only resistant strains were isolated. Not only did sulfadiazine treatment fail to influence the actual number of variants with decreased resistance, but it appeared to have no relation to the serological types in which these variants occurred. Thus 7 of the 9 patients from whom variant strains were obtained were infected with type 19 streptococci, one had a type 3, and another a type 26 infection. Similarly, patients with consistently resistant strains also had infections with a variety of serological types: 11 had type 19 infections, 2 type 30, and one each type 6, 18, or 38 infections.

Although an insufficient number of strains of the various serological types were available to determine whether this variation has any relationship to the type or is characteristic of particular strains, some of the data may be significant in this respect. Of the 21 series of type 19 strains studied, 20 were isolated from individual patients who developed scarlet fever in the same epidemic (13). In 7 of these 20 patients, type 19 variants which proved susceptible to bacteriostasis appeared during convalescence; while from the remaining 13 patients, only resistant strains were isolated during the period of observation. Although in several other types all the strains were on the contrary alike in resistance or susceptibility, the numbers in each type were small: the strains recovered from the 3 patients with type 26 infections all developed susceptible variants; and only resistant strains were recovered from the 3 patients with type 30 infections and the 2 patients infected with type 12 streptococci. In the remaining types some strains remained resistant throughout the study of the patient and some in each serological type encountered became susceptible. Consequently, no correlation could be established between these strain variations and the serological types involved.

Todd and Lancefield (6) have demonstrated that mouse-virulent matt variants of group A streptococci can be converted to mouse-avirulent glossy variants by repeated subculture in media containing homologous type-specific rabbit antisera. It appeared of interest, therefore, to determine whether any relation existed between the occurrence of variation among these strains and the appearance of type-specific antibodies in the sera of patients from whom the streptococci were obtained. In a previous study (14) reported elsewhere, type-specific bacteriostatic antibody determinations had been made on the sera of 37 patients whose series of strains were studied in this present investigation. Both resistant and susceptible strains were found among patients who developed bacteriostatic antibodies: 16 patients had resistant strains and 15 had susceptible strains. Among the remaining 6 patients with no type-specific antibody response, susceptible variants appeared in every case. It is, therefore, apparent that there was no correlation between the appearance of susceptible

variants and type-specific antibody formation. Moreover, no correlation was found between the time of appearance of the variant strains and the formation of antibodies, nor was there any relation between the height of the antibody response and the degree of variation. Apparently, the variation of these streptococcal strains cannot be explained by the concomitant appearance of type-specific bacteriostatic antibodies.

Since 23 of the 56 streptococcal infections were followed by complications, an attempt was made to ascertain whether there was any correlation between the appearance of this variation phenomenon and the development of these complications, but no such relationship was found. Of the 23 complicated infections, strains which showed some degree of variation were recovered from 11 (3 purulent and 8 rheumatic fever), and only stable strains were isolated from the remaining 12 (3 purulent and 9 rheumatic fever). As far as could be determined, the complications appeared in all instances before the isolation of the variant strains of streptococci. From the 33 remaining infections which were uncomplicated, 12 yielded strains which showed variation and 21 yielded strains which showed no change.

Relation of the Type-Specific M Protein Antigen to Bacteriostasis.—In a previous study (12) it has been shown that bacteriostatic susceptible variants produce less M protein than do the parent resistant strains. To determine whether this relationship held in the present study, the 251 strains were also tested for their capacity to produce the M protein antigen. From 4 patients (4 infections), 17 strains were not included because they did not fall into known serological types. From the remaining 50 patients with 52 infections, 234 strains of known serological types were tested. In 42 per cent of the 52 infections, strains isolated in the convalescent and carrier stages showed an increasing susceptibility to bacteriostasis correlated with a progressive loss of M substance; whereas in the remaining 58 per cent, resistance to bacteriostasis and the capacity to produce M protein were maintained throughout the observation period.

In Table III are recorded the results of titration for the M protein antigen prepared from the highly resistant strain recovered on the initial culture and the less resistant strain isolated on the last culture. The streptococcal extracts containing M protein antigen were diluted in a twofold serial manner, and tested with type-specific absorbed rabbit antisera. The data in Table III reveal that the susceptible variant strain in each series produced less M substance by at least a 2-tube dilution difference than did the highly resistant parent strain; 4 of the strains showed a 4-tube dilution difference; 5, a 5-tube difference; 2, a 6-tube and 1, a 7-tube dilution difference. Similar titrations were also done on each of the series of stable strains which showed no evident variation, but these results are not tabulated because no difference was found in the amount of M substance produced by the strains in each respective series.

From 3 patients (one with a type 14 infection and 2 with type 26 infections),

TABLE III

Production of Type-Specific M Substance of Group A Streptococci Highly Resistant to Bacteriostasis as Compared to Their Susceptible Variants

Serological type	Bacteriostatic variant			
	Highly resistant		Susceptible*	
	Wk. isolated	M titre‡	Wk. isolated	M titre‡
1	1	1:32	12	1:2
1	1	1:8	5	1:1
3	1	1:16	16	1:4
3	1	1:32	5	1:1
6	1	1:32	20	1:4
6	1	1:16	14	1:2
6	1	1:16	9	1:4
14	1	1:32	11	0
17	1	1:16	55	1:4
19	1	1:32	9	1:4
19	1	1:16	10	1:4
19	1	1:32	9	1:8
19	1	1:64	7	1:2
19	1	1:32	6	1:2
19	1	1:32	7	1:2
19	1	1:64	6	1:2
26	1	1:64	21	0
26	1	1:64	23	1:2
26	1	1:32	19	0
29	1	1:32	4	1:1
33	1	1:32	8	1:2
38	1	1:8	7	1:1

* Indicates variants which show any degree of diminution of resistance from original highly resistant strains.

‡ Titre signifies highest dilution of type-specific M protein extract which gave a positive reaction with homologous rabbit antiserum.

strains were recovered which were so degraded that no M protein could be demonstrated in acid extracts of these variants by the precipitin technique. It is possible that others would have become degraded to a similar degree had the patients been followed longer. In Tables IV *a*, IV *b*, and IV *c* are recorded the

degrees of resistance to bacteriostasis and the M titre for every strain isolated in series from each of these 3 patients. From Table IV *a* it is evident that at about the 8th week after the onset of a type 14 infection a beginning loss of resistance to bacteriostasis occurred but with little apparent variation in M protein synthesis; however, during the 9th week, a further decrease in resistance to bacteriostasis occurred and for the first time a definite decrease in the titre of M was noted. By the 11th week there was little resistance to bacteriostasis and no M substance was demonstrable on precipitin test. After 25 passages through mice, the capacity of this degraded variant to resist the bacteriostatic action of normal human blood and to produce the M protein substance was restored to the levels noted on primary isolation.

From Tables IV *b* and IV *c* it can be seen that strains degraded to a similar degree were recovered from the other 2 patients. Unfortunately the cultures taken on one of these patients between the 3rd and 21st week revealed no streptococci so that the gradual change is not shown in Table IV *c*. The most degraded variants in these 2 series also regained after mouse passage their capacities to elaborate the M substance and to resist the bacteriostatic action of human blood.

The series of strains shown in Table IV *c* is of particular interest because special means were available for identification of the variant strain, 24 RS50, and the original strain, 11 RS50. This latter strain was unusual in two respects: It lacked the T antigen found in most type 26 strains; and it had a peculiar and characteristic T antigen which has so far not been observed in any other strain studied in this laboratory. Because after an interval of 18 weeks, during which time no group A streptococci were obtainable on serial weekly cultures, the group A streptococci recovered from this patient no longer synthesized type 26 M substance, a special study was undertaken to determine whether the peculiar T antigen of the original strain was still present in the later or variant strain. These 2 strains were used to immunize respective groups of rabbits, and antibodies specific for the peculiar T antigen of the original strain 11 RS50 were demonstrated in the sera of both groups of rabbits by direct agglutination reactions and specific cross-absorption of the 2 kinds of antisera with the 2 strains studied. Control absorption tests with type 26 strains, which contained the usual T antigen of type 26, failed to remove the T antibodies peculiar to the RS50 series of strains. Finding this unusual T antigen in both of these strains is additional evidence that the variant strain 24 RS50 was actually a derivative of the original strain 11 RS50 isolated from this patient.

Moreover, by means of agglutination tests with rabbit antisera directed toward the type 14 and type 26 T antigens respectively, it was also possible to show that the variants listed in Tables IV *a* and IV *b* had the same T antigens as those in the original strains isolated from each of these patients.

The degraded variants in these 3 series of strains appear to be relatively stable

TABLE IV *a*

Resistance to Bacteriostasis Correlated with Production of Type-Specific M Protein Antigen by Group A Type 14 Streptococci Isolated from a Patient with Acute Pharyngitis

Streptococcal strain No.	Serological type	Wk. of infection strain isolated	Bacteriostasis of streptococcal strains: Dilution of culture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titration of type-specific M extract: Dilution of extract 1:1	1:2	1:4	1:8	1:16	1:32	1:64
23 RS84	14	1	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
24 RS84	14	2	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
25 RS84	14	3	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
26 RS84	14	4	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
27 RS84	14	5	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
28 RS84	14	6	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
29 RS84	14	7	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0
32 RS84	14	8	++++	++++	++++	+++	++	0	+++	+++	++	++	++	+	0
33 RS84	14	9	++++	++++	++++	++±	9	0	+++	+++	++	+	±	0	0
34 RS84	14	10	++++	++++	+++	++	0	0	+++	+++	++	+	±	0	0
35 RS84	14	11	++	2	0	0	0	0	0	0	0	0	0	0	0
35 RS84/25/O*	14	—	++++	++++	++++	++++	+++	++	+++	+++	++	++	++	+	0

In bacteriostasis of strains, degree of growth is indicated on a ++++ to + scale; fewer than 10 colonies are represented in arabic numerals; 0 indicates no growth.

In titration of M antigen, the degree of precipitation is indicated on a ++++ to ± scale; 0 represents no precipitation.

* This strain was passed through mice 25 times and regained its capacity to produce the M substance.

TABLE IV *b*

Resistance to Bacteriostasis Correlated with Production of Type-Specific M Protein Antigen by Group A Type 26 Streptococci Isolated from a Patient with Acute Pharyngitis

Streptococcal strain No.	Serological type	Wk. of infection strain isolated	Bacteriostasis of streptococcal strains: Dilution of culture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titration of type-specific M extract: Dilution of extract 1:1	1:2	1:4	1:8	1:16	1:32	1:64
14 RS15	26	1	++++	++++	++++	++++	+++	++	++±	++	++	++	++	+	0
15 RS15	26	2	++++	++++	++++	++++	+++	++	++±	++±	++	++	++	+	0
16 RS15	26	3	++++	++++	++++	++++	+++	++	++	++	++	++	++	+	0
17 RS15	26	4	++++	++++	++++	++++	+++	++	++	++	++	++	++	+	0
18 RS15	26	5	++++	++++	++++	++++	+++	++	++	++	++	++	++	±	±
19 RS15	26	6	++++	++++	++++	++++	++	+	++	++	++	++	+	±	0
20 RS15	26	7	++++	++++	++++	++++	+	8	++	++	++	++	±	±	0
21 RS15	26	8	++++	+++	++±	++	0	0	++	++	++	+	0	0	0
22 RS15	26	14	+	7	0	0	0	0	±	0	0	0	0	0	0
23 RS15	26	19	+	0	0	0	0	0	0	0	0	0	0	0	0
23 RS15/25/O*	26	—	++++	++++	++++	++++	+++	++	+++	+++	+++	++	++	+	0

Same symbols for bacteriostasis of strains and titration of M antigen as used in Table IV *a*.

* This strain was passed through mice 25 times and regained its capacity to produce the M substance.

TABLE IV *c*

Resistance to Bacteriostasis Correlated with Production of Type-Specific M Protein Antigen by Group A Type 26 Streptococci Isolated from a Patient with Acute Pharyngitis

Streptococcal strain No.	Serological type	Wk. of infection strain isolated	Bacteriostasis of streptococcocal strains: Dilution of culture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titration of type-specific M extract: Dilution of extract 1:1	1:2	1:4	1:8	1:16	1:32	1:64
11 RS50	26	1	++++	++++	++++	++++	++++	+++	+++	++±	++±	++	+	+	±
12 RS50	26	2	++++	++++	++++	++++	+++	+++	+++	+++	++	++	+	±	0
13 RS50	26	3	++++	++++	++++	++++	+++	++	+++	+++	++	++	+	+	0
24 RS50	26	21	++++	++	7	0	0	0	0	0	0	0	0	0	0
24 RS50/21/O*	26	—	++++	++++	++++	++++	+++	++	+++	+++	++	++	+	±	0

Same symbols for bacteriostasis of strains and titration of M antigen as used in Table IV *a*.

* This strain was passed through mice 21 times and regained its capacity to produce the M substance.

antigenically since no evidence of spontaneous reversion to the original state has been noted after several years. It may also be noteworthy that only 2 of the 3 patients from whom these degraded variants were isolated developed type-specific bacteriostatic antibodies following infection. This indicates, as previously noted, that the production of type-specific bacteriostatic antibodies does not explain the variation.

The colony morphology was carefully investigated on fresh sheep and rabbit blood agar plates to see whether any difference could be observed by this means between the bacteriostatic-resistant M-producing parent strain and the degraded bacteriostatic-susceptible non-M-producing variant. No significant difference was detected, nor was capsule formation of any help in differentiating the 2 variants of the various strains.

In an attempt to explain the loss of M antigen, the variants which completely lost their ability to produce M were repeatedly tested for the production of streptococcal proteinase, an extracellular proteolytic enzyme which digests the M protein (15). This proteolytic enzyme could not be demonstrated in cultures of any of the parent strains or their variants.

These data show that group A streptococci may lose their capacity to elaborate the type-specific M protein antigen and become susceptible to the bacteriostatic action of human blood while the bacteria are residing in the host's tissues; but the factor or factors which are responsible for the occurrence of these phenomena are still unknown.

DISCUSSION

From the evidence presented in this report, it appears that in the course of group A streptococcal infections in man, variant strains often occur which are characterized by a decreased capacity to produce the type-specific M protein antigen, and a concomitant decrease in resistance to the bacteriostatic action of normal human blood. This variation occurs gradually over a period of weeks during convalescence and the carrier period while the streptococci persist in the nasopharyngeal tissues of the host. In the 3 series of strains each characterized by the development of variants which lost their capacity to produce the M antigen, the same T antigen was retained in each instance as that present in the strain originally isolated from the patient. These degraded variants are relatively stable since reversion after numerous subcultures on bacteriological media has not occurred; the original capacities, however, to produce the M substance and to resist bacteriostasis can be restored by serial mouse passage. Since the T antigen was found to be similar in both the variant and original strains, and since the M-producing capacity was restored to the variant on mouse passage, it appears that these degraded strains were derived from the original strains which caused the patient's infection.

In seeking an explanation for the variation, it was found that its occurrence

was not related to the production of type-specific antibody by the host, nor was it dependent on the production by the variants of a proteolytic enzyme (15) which is known to destroy the M protein.

The possibility that the degraded variants were present at all times during the infection and that in convalescence they were selected on culture by chance seems very unlikely; nevertheless this must be considered. The fact that multiple colonies picked from a single culture failed to reveal differences in variation seems to be against this possibility. Moreover, the gradual and progressive changes only in the direction of further degradation also suggest that no chance selection occurred.

The decrease in resistance of streptococcal strains to bacteriostasis by normal blood has uniformly been associated with a diminished capacity of the streptococcal cells to produce the M protein antigen. The M substance, which is probably a surface antigen, has been shown to play an important rôle in the exhibition of virulence, and in these respects is analogous to the type-specific polysaccharide of the pneumococcus. From the evidence at hand it would appear that the M protein plays an important part in protecting the streptococcal cell from phagocytosis by the leukocytes of normal blood. In some instances the decreased resistance to bacteriostasis was evident before a definite decrease in the capacity of the streptococcal cells to produce the M protein could be demonstrated. This is probably explained by a difference in the sensitiveness of the two tests: the bacteriostatic test being more sensitive for demonstrating a slight loss of the M substance than the precipitin test.

Although knowledge of the factor or factors which are responsible for the occurrence of variation during the natural course of group A streptococcal infections remains obscure, there is ample evidence to show that this phenomenon does occur frequently enough possibly to be of considerable importance from the standpoint of preventive medicine. There is some evidence to indicate that certain persons harboring hemolytic streptococci are more likely to disseminate infection than others (16, 17). It is possible that among other factors which make one host less dangerous than another is the fact that in some the strains may undergo variation with an associated decrease in virulence and invasiveness. What perhaps is more important is that the majority of strains do not lose their capacity to synthesize the M protein for relatively long periods after the onset of infection and are therefore potentially dangerous pathogens.

SUMMARY

A study was made of the variation occurring in group A streptococci during the natural course of infection in man. From 54 patients with 56 different group A streptococcal infections of the upper respiratory tract, 251 strains of streptococci, isolated at weekly intervals following infection, were tested for their capacity to resist the bacteriostatic action of normal human blood. In 52 of the infections the streptococci were of recognized serological types and were

also tested for variation in their ability to produce the type-specific M protein antigen. Strains isolated in the 1st week of infection were uniformly highly resistant to bacteriostasis and elaborated large amounts of M substance. In 42 per cent of the 52 infections, strains isolated in the convalescent and carrier stages showed an increasing susceptibility to bacteriostasis correlated with a progressive loss of M substance; whereas in the remaining 58 per cent resistance to bacteriostasis and the capacity to produce M protein were maintained throughout the observation period.

In 3 different infections, the streptococci became so degraded that no M protein could be demonstrated in acid extracts of these variants. Concomitantly these strains became highly susceptible to bacteriostasis. Spontaneous reversion did not occur, but serial mouse passage reestablished these functions. These degraded variants had the same T antigen as their respective original strains.

No evidence was obtained that variation of group A streptococci in resistance to bacteriostasis or in the ability to produce the type-specific M antigen was associated (*a*) with the appearance of type-specific bacteriostatic antibodies; (*b*) with any particular serological type of streptococcus; (*c*) with the production of streptococcal proteinase which digests the M protein; (*d*) with the therapeutic administration of sulfadiazine; or (*e*) with the development of complications.

The possible relationship of these observations to the problem of the "dangerous carrier" of group A hemolytic streptococci is discussed.

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THE REVERSIBILITY OF THE O-D TYPE OF INFLUENZA VIRUS VARIATION*‡

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It is still a matter of conjecture whether the influenza virus consists of a large number of stable but antigenically different strains; or whether that agent is a relatively unstable one, the antigenic properties of which are subject to changes of an unknown nature. It has been suspected for years by some workers that the virus is subject either to variation, or to mutation; and those suspicions have been increased by the recent rather sudden appearance of strains which differ sharply in antigenic properties from previously recognized strains. The potential epidemiological significance of the sudden appearance of variants, or mutants, is obvious; but conclusive experimental evidence in support of the idea of variation or mutation has been lacking. However, some data have been reported:

Several years ago Burnet and Bull (1) reported that strains of influenza A virus may exist in 2 forms, one of which—termed "O" or "original" by those authors—agglutinated guinea pig but not chicken erythrocytes; and the other of which—termed "D" or "derived"—agglutinated guinea pig and chicken erythrocytes to approximately the same degree: the 2 forms differed in tropism; the O form could be maintained only by amniotic passage of infected chick embryo lung and trachea, whereas the D form could be maintained by either allantoic or amniotic passage of infected fluids. Those findings (which were partially confirmed by Hirst in reports (2, 3) which appeared during the course of the present investigation) enhance the evidence presented earlier by Stuart-Harris (4) and confirmed by Francis and Moore (5), that the tropism of influenza virus is subject to change.

The data included in the present paper concern the O-D type of change. They are significant from 2 aspects: (1) They indicate that the change is not a discontinuous mutation as suggested by Burnet and Bull (1), but is a readily reversible variation. (2) They show, quite clearly, that the inability of the O form of virus to agglutinate chicken erythrocytes under usual test conditions is a relative phenomenon associated with physical-chemical factors; that under

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suitable test conditions the O form has an affinity for chicken as well as for guinea pig erythrocytes.

Materials and Methods

Virus.—The WS (6) strain of influenza A virus was selected because its tropism is known to be subject to change: Stuart-Harris (4) showed that a variant with neurotropic properties could be derived from the usual pneumotropic strain, and his findings were confirmed by Francis and Moore (5).

Egg Inoculations.—Amniotic passage was made in eggs containing 11 or 12 day old embryos: the shell (cleaned with iodine and alcohol) was removed from the area over the airspace, and the chorio-allantoic membrane exposed in order to visualize the amniotic sac and embryo. Inoculations were made with 1 inch, 23 gauge hypodermic needles, and the open ends of the eggs then were covered with 38 × 20 mm. staining dishes.

Allantoic passage was performed by inoculating the material beneath the chorio-allantoic membrane through a hole in the shell; ⅜ of an inch, 26 gauge needles were used; holes were sealed with paraffin.

Passage material contained sufficient penicillin to provide about 200 units per egg. Incubation was at 35°C. or 37°C.

In Vitro Methods of Cultivation.—The methods employed were similar to one described previously (7). Three sources of cells from embryonated hen's eggs were utilized,—chorio-allantoic membrane, embryo lung, and whole embryo. To each 50 ml. Erlenmeyer flask containing 4 or 4½ ml. of saline and 200 units of penicillin, was added approximately half of a chorio-allantoic membrane (from eggs containing 12 to 16 day embryos) cut into pieces ½ to 1 cm. in size; the minced lungs from several 14 to 16 day old embryos; or approximately ½ of a whole minced 12 to 13 day old embryo. Incubation was at 35°C.

Hemagglutination.—Tests for agglutination of erythrocytes were made after the method described by Hirst (8), in 10 × 75 mm. precipitin tubes in which the total volume of ingredients was 0.6 ml. The degrees of agglutination were determined by the cell patterns on the tube bottoms, after the tests had been at room temperature sufficiently long (about 2 hours) for the erythrocytes to settle completely; they are recorded as no (0), partial (+), and complete (++) agglutination.

The erythrocyte suspensions employed were approximately ½ per cent by volume, prepared from thoroughly washed chicken or guinea pig cells obtained aseptically.

RESULTS

In preliminary experiments with Mel A and Ian A strains[1] we were able to confirm the findings of Burnet and Bull (1) concerning the existence of 2 "phases" in a single strain of influenza virus, one of which (O, or original) has much greater hemagglutinating activity against guinea pig than against chicken erythrocytes, and the other of which (D, or derived) agglutinates the erythrocytes of those 2 species to approximately the same degree.

It was our experience, however, that during selective amniotic passage of infected embryo lung and trachea, the O phase might abruptly revert to the D phase, or the D might revert to the O. The derivation of either of the 2 forms appeared to be haphazard and subject to chance. The D form was the more

[1] Received from Dr. F. M. Burnet.

dominant of the 2, even when dilute (10^{-5}) infected embryo lung and trachea was passaged every 48 hours amniotically (the method by which Burnet and Bull (1) maintained their O form). Nevertheless, when a sufficiently large number of eggs (10 to 12) was employed in each passage, amniotic passage of infected lung resulted, within 4 or 5 passages, in the reversion of the D to the O form in one or more amniotic fluids. That is, the O and D forms each appeared to have an inherent capacity to give rise to the other.

In those preliminary experiments with Mel A and Ian A strains, we obtained the O phase of the virus only by amniotic passage of infected lung and trachea; amniotic passage of allantoic fluid or of chorio-allantoic membrane rather uniformly yielded amniotic fluid which showed the D form of hemagglutination. Thus the data are in agreement with the findings of Burnet and Bull that the O and D phases are associated in some manner with different tropisms.

Derivation of O Form of WS Strain of Virus from Mouse Passage Material.—The foregoing experiments indicate clearly that the O-D change is a reversible one and suggest that the O form might be derived from an established laboratory D form strain. The following experiment was made to test this point:

The WS strain of virus in the form of infected mouse lung which had been preserved in the dried state for over 6 years was reconstituted in distilled water and diluted 10^{-2} with infusion broth; penicillin was added and 6 eggs containing 12 day old embryos were inoculated,—three into the amniotic sac, and three beneath the chorio-allantoic membrane. After 5 days at 37°C. lung and trachea from one of the amniotically inoculated eggs was removed and ground without abrasive in 2 ml. of sterile distilled water; the resultant suspension was diluted 10^{-2} with infusion broth, penicillin was added, and a series of eggs containing 12 day old embryos was inoculated amniotically. After 4 days at 37°C. the amniotic fluids were tested with chicken and guinea pig erythrocytes for hemagglutinating activity, and the embryo from the egg, the amniotic fluid of which showed the best O form hemagglutination, was selected for passage. The agglutinating capacities of amniotic fluids from 4 such serial passages are shown in Table I.

The data (Table I) show that the WS strain of influenza virus (that previous to storage in the dried state had been maintained only in mice for more than 100 passages) was propagated readily by amniotic passage; and that amniotic passage of selected embryo lung and trachea resulted in rapid derivation of the O form from the D. Moreover, they illustrate the suddenness with which either form may revert to the other. In passage 2 (actually the first passage of infected lung and trachea) the amniotic fluid of one (No. 2) of the 4 eggs inoculated showed marked O form hemagglutination; and on the very next passage 7 of the 8 eggs tested showed O form hemagglutination. However, there was sudden reversion to the D form on the following passage (No. 4).

The influence of tropism upon the O-D change of the WS strain is shown by data not included in Table I: during allantoic passage of infected chorio-allantoic membrane, only the D form appeared in the allantoic fluids tested. That

TABLE I

Derivation of the O Phase of the WS Strain of Influenza Virus by Means of Amniotic Passage of Infected Embryo Lung and Trachea

Passage No.	Egg No.	RBC*	Hemagglutination: Twofold dilution of amniotic fluid													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1	ch	++	++	++	++	++	++	++	++	+	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
	2	ch	++	++	++	++	++	++	++	++	++	++	+	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
	3‡		Not tested													
2	1	ch	++	++	++	++	++	++	++	++	++	+	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
	2‡	ch	++	++	+	+	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	+	0	0	0
	3	ch	++	++	++	++	++	++	++	++	++	+	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
	4	ch	++	++	++	++	++	++	++	++	++	++	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
3	1	ch	++	0	0	0	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	+	0	0	0
	2	ch	+	+	+	0	0	0	0	0	0	0	0	0	0	
		gp	++	++	++	++	++	++	++	++	++	++	++	++	+	0
	3	ch	+	+	+	+	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	+	0	0
	4	ch	++	++	++	+	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	+	+	0	0
	5	ch	++	++	++	++	++	++	++	+	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	+	0	+	0	0	0
	6	ch	+	0	0	0	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	0	0	0	0
	7	ch	+	+	++	+	+	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	++	+	0

TABLE I—*Concluded*

Passage No.	Egg No.	RBC*	Hemagglutination: Twofold dilution of amniotic fluid													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
	8‡	ch	+	0	0	0	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	++	+	0
4	1	ch	++	++	++	++	++	+	+	+	+	0	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	++	++	+
	2	ch	++	++	++	++	++	++	++	++	++	+	0	0	0	0
		gp	++	++	++	++	++	++	++	++	++	++	++	++	+	+
	3	ch	++	++	++	++	++	++	++	++	++	+	0	0	0	0
		gp	++	++	++	++	++	++	++	++	+	+	0	0	0	0
	4	ch	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		gp	++	++	++	+	0	0	0	0	0	0	0	0	0	0

++, complete agglutination; +, partial agglutination· 0, no agglutination.

* ch, chicken erythrocytes; gp, guinea pig erythrocytes.

‡ Egg from which passage material (embryo lung and trachea) was taken.

is, the D form was propagated by both methods tested, but the O form occurred only following amniotic passage of infected embryo lung and trachea.

Derivation of O from D phase Virus by in Vitro Cultivation with Cells of the Chorio-Allantoic Membrane.—The following experiment was made to determine the influence of *in vitro* cultivation with cells of a single variety upon propagation of the O and D forms.

Two series of cultures were started: O phase WS strain of virus that had been derived by selective amniotic passage of infected embryo lung, was passaged in flasks containing minced embryo lung; and D phase virus that had been maintained by allantoic passage of infected chorio-allantoic membrane, was passaged in flasks containing chorio-allantoic membrane. The virus survived no more than 3 or 4 transfers in flasks containing embryo lung but was propagated readily in flasks containing chorio-allantoic membrane. Minced whole chick embryo was substituted for embryo lung, and 2 series of cultures were maintained with cells from both sources (chorio-allantoic membrane and whole embryo). In one series, transfers were made every 24 hours with 1 ml. of undiluted supernatant fluid (*i.e.*, the virus suspension was diluted $\frac{1}{2}$ on each transfer); in the other series, transfers were made every 48 hours with 0.5 ml. of 10^{-2} dilution of supernatant fluid (*i.e.*, the virus suspension was diluted 10^{-3} on each transfer).

The results of the experiments were surprising in that many supernatant fluids from the *in vitro* chorio-allantoic membrane cultures agglutinated guinea pig erythrocytes much more readily than chicken erythrocytes (O form hemag-

glutination). Furthermore, allantoic fluids from many eggs inoculated allantoically with fluids from the *in vitro* chorio-allantoic membrane cultures exhibited clear-cut O form hemagglutination.

Table II includes the results of tests for agglutination of chicken and guinea pig erythrocytes by allantoic fluids from eggs which had been inoculated with supernatant fluids from one group of *in vitro* cultures of WS strain of virus: in one series (24 hour passage) the cultures had been through 35 serial transfers, and in the other (48 hour passage) through 20 serial transfers. The left hand column (table II) shows the source of cells employed in the *in vitro* cultures from which the inoculation fluids had been obtained, and the dilution of these culture fluids with which the respective test eggs had been inoculated. It is clear (Table II) that the O form of the WS strain of virus appeared in allantoic fluids from eggs inoculated with fluids from cultures containing cells of the chorio-allantoic membrane, perhaps even more frequently than in the allantoic fluids from eggs inoculated with fluids from cultures containing minced embryo. The O phase hemagglutination is especially evident in tests with allantoic fluids 1, 7, 8, 9, 10, 20, and 26 (Table II).

It is of interest that in this particular group of tests, the O form hemagglutination occurred more frequently with allantoic fluids of eggs inoculated with the various dilutions of fluids from both the 48 hour (10^{-3} dilution) passage cultures than with those from eggs inoculated with fluids from the 24 hour (1/5 dilution) passage cultures. That point supports the data of Burnet and Bull (1) that the D phase of the virus occurs considerably less frequently but multiplies more rapidly than the O phase. It should be noted, however, that Burnet and Bull failed to obtain O phase virus after repeated passage of concentrated virus suspensions; whereas in the present experiment O phase virus occurred in allantoic fluid 1 (Table II) which was from an egg inoculated with undiluted supernatant fluid from the 35th, 24 hour passage, each passage of which represented only a 1-5 dilution of virus suspension. That fact clearly emphasizes the inherent O phase trait within the D phase virus.

During the present and other tests, we frequently encountered fluids which produced rather clear-cut agglutination of guinea pig erythrocytes, but which produced partial agglutination of chicken erythrocytes. That partial agglutination of chicken cells was fluffy in appearance and the agglutination endpoint was difficult to determine; presumably, it was similar to that recently described by Hirst (3). Agglutination of that kind is indicated by an asterisk in Table II. In the tests included in Table II similar fluffy agglutination sometimes occurred with guinea pig erythrocytes, but whenever it did occur it did so in tests with the more concentrated allantoic fluid,—indicating inhibition of the agglutination. Such inhibition of agglutination of guinea pig erythrocytes is evident in tests with allantoic fluids 1, 10, 14, 21, and 22. The peculiar nature, in some instances, of chicken erythrocyte agglutination and the sug-

TABLE II

Hemagglutination by Allantoic Fluids from Eggs Inoculated with WS Strain of Virus Cultivated in Vitro

Material with which test eggs were inoculated		Allantoic fluid No.	RBC*	Hemagglutination by allantoic fluids from test eggs							
				Threefold dilution of allantoic fluid							
Tissue used in culture	Dilution of culture fluid			1	2	3	4	5	6	7	8
Chorio-allantoic membrane 24 hr. passage	10^{0}	1	ch	0	0	0	0	0	0	0	0
			gp	+‡	+	++	++	++	0	0	0
	10^{-2}	2	ch	+‡	+‡	+‡	0	0	0	0	0
			gp	+	++	++	++	+	0	0	0
	10^{-3}	3	ch	++‡	++‡	+	+	0	0	0	0
			gp	+	++	++	++	++	+	0	0
	10^{-4}	4	ch	+‡	+‡	+‡	+‡	0	0	0	0
			gp	++	++	++	++	+	0	0	0
	10^{-5}	5	ch	++	++	+	0	0	0	0	0
			gp	++	++	++	++	++	++	0	0
	10^{-6}	6	ch	++	++	++	++	++	+	0	0
			gp	++	++	++	++	++	+	0	0
Chorio-allantoic membrane 48 hr. passage	10^{0}	7	ch	0	0	0	0	0	0	0	0
			gp	++‡	++	++	++	++	+	0	0
	10^{-1}	8	ch	+‡	0	0	0	0	0	0	0
			gp	++	++	++	++	++	+	0	0
	10^{-2}	9	ch	+‡	0	0	0	0	0	0	0
			gp	+‡	++	++	++	++	+	0	0
	10^{-3}	10	ch	+‡	0	0	0	0	0	0	0
			gp	++‡	+	+	+	++	++	++	++
	10^{-4}	11	ch	+‡	+‡	+	++	+	0	0	0
			gp	++	++	++	++	++	++	0	0
	10^{-5}	12	ch	+‡	+‡	0	0	0	0	0	0
			gp	+‡	++	++	++	++	++	0	0
	10^{-6}	13	ch	+‡	+‡	0	0	0	0	0	0
			gp	+‡	++	++	++	++	++	+	0

TABLE II—*Concluded*

Material with which test eggs were inoculated		Allantoic fluid No.	RBC*	Hemagglutination by allantoic fluids from test eggs							
				Threefold dilution of allantoic fluid							
Tissue used in culture	Dilution of culture fluid			1	2	3	5	5	6	7	8
Embryo 24 hr. passage	10^{0}	14	ch	+‡	+‡	+	+	+	0	0	0
			gp	+‡	+‡	++	++	++	++	+	0
	10^{-1}	15	ch	+‡	+	+	+	+	0	0	0
			gp	++	++	++	++	++	++	0	0
	10^{-2}	16	ch	+‡	+‡	+	+	+	0	0	0
			gp	+‡	++	++	++	++	0	0	0
	10^{-3}	17	ch	+‡	+‡	+‡	+	+	0	0	0
			gp	+‡	++	++	++	++	0	0	0
	10^{-5}	18	ch	+‡	+‡	+	+	+	0	0	0
			gp	++	++	++	++	++	++	0	0
	10^{-6}	19	ch	0	0	0	0	0	0	0	0
			gp	0	0	0	0	0	0	0	0
Embryo 48 hr. passage	10^{0}	20	ch	0	0	0	0	0	0	0	0
			gp	+	++	++	++	++	++	+	0
	10^{-1}	21	ch	++	++	+	+	+	0	0	0
			gp	+	+	++	++	++	++	+	0
	10^{-2}	22	ch	++	+	0	0	0	0	0	0
			gp	+	+	++	++	++	++	0	0
	10^{-3}	23	ch	++	+	0	0	0	0	0	0
			gp	++	++	++	++	++	++	+	0
	10^{-4}	24	ch	++	+	0	0	0	0	0	0
			gp	+	++	++	++	++	++	+	0
	10^{-5}	25	ch	0	0	0	0	0	0	0	0
			gp	++	+	0	0	0	0	0	0
	10^{-6}	26	ch	+	0	0	0	0	0	0	0
			gp	++	++	++	++	++	++	+	0

* ch, chicken erythrocytes; gp, guinea pig erythrocytes.

‡ Agglutination of a peculiar "fluffy" appearance.

gested inhibition of guinea pig erythrocyte agglutination aroused the suspicion that the O form of hemagglutination might be associated with agglutination inhibition by substances present in the test system.

Change of O Form Hemagglutination to the D Form by Means of Adjustment of the pH of the System.—In a previous report (9) we showed that the failure of the WS strain of influenza A virus to agglutinate sheep erythrocytes under usual

TABLE III

O Phase Hemagglutination Changed to D Phase by Adjustment of the pH of the System

Allantoic fluid No.*	RBC†	Hemagglutination by allantoic fluids															
		pH of system unadjusted								pH of system adjusted to pH 5.6 with buffer§							
		Threefold dilution of fluid								Threefold dilution of fluid							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	ch	+	0	0	0	0	0			++	++	++	++	+	0		
	gp	+	++	++	++	++	+	0	0	++	++	++	++	++	++	0	
2	ch	+	+	+	0	0	0	0		++	++	++	++	+	0		
	gp	++	++	++	++	++	0	0		++	++	++	++	++	0	0	
7	ch	0	0	0	0	0	0			++	++	++	++	++	0		
	gp	++	++	++	++	++	++	0	0	++	++	++	++	++	+	0	
9	ch	+	+	0	0	0	0			++	++	++	++	++	+		
	gp	++	++	++	++	++	++	0	0	++	++	++	++	++	++	0	
20	ch	0	0	0	0	0	0			++	++	++	++	++	++		
	gp	++	++	++	++	++	++	0	0	++	++	++	++	++	++	+	0
26	ch	+	0	0	0	0	0			++	++	++	++	++	++		
	gp	++	++	++	++	++	++	0	0	++	++	++	++	++	++	0	0

* Allantoic fluids same as those (of same number) included in Table II.

† ch, chicken erythrocytes; gp, guinea pig erythrocytes.

§ Buffer, McIlvaine phosphate-citric acid.

test conditions was due, not to a lack of affinity of the virus for erythrocytes, but rather to factors in the test system which inhibited agglutination; and when those factors were controlled, the virus readily agglutinated sheep erythrocytes. In view of those findings, the indications that the O form hemagglutination might be associated with inhibition, suggested that those inhibitory substances might also be controlled through adjustment of the pH of the test system. The following experiment was made to test this point:

The allantoic fluids listed in Table II, which showed the most marked O phase hemagglutination were tested in 2 series. One series of tests was made in the

usual manner with 0.2 ml. quantities of allantoic fluid (diluted in saline), saline, and erythrocytes (suspended in saline). The other series differed in that one volume of buffer replaced the volume of saline. The buffer used was McIlvaine's phosphate-citric acid (10); it was selected because we have found it to be superior to phosphate, and to acetate buffers for the purpose. The selection of pH 5.6 was made because in unpublished experiments we have found that, with appropriate suspensions of strains of influenza A virus, agglutination of chicken erythrocytes was influenced to a considerable degree by the pH of the test system; agglutination was best at pH 5.6.

Table III includes the results of the 2 series of tests. The data show in a rather striking manner that when tested under usual conditions, the allantoic fluids caused little or no agglutination of chicken erythrocytes but rather marked (3^6) agglutination of guinea pig erythrocytes. But when the pH of the test system was adjusted to pH 5.6 (McIlvaine's buffer), the same allantoic fluids agglutinated the same 2 erythrocyte suspensions to approximately the same degree.

DISCUSSION

The O-D type of change in characteristics of strains of influenza virus, described by Burnet and Bull (1), is important because it furnishes additional evidence that the influenza virus is not a stable agent. Although such evidence does not contribute directly to our knowledge concerning antigenic differences among strains of influenza virus, it does sustain the suspicion that the existence of so many antigenically related but different strains may be the result of variability of the virus. Burnet and Bull (1) concluded that the change is a discontinuous mutation and indicated it graphically as O→D, obviously inferring that it occurs in but one direction. The title and content of their paper indicate that the change is the result of adaptation. The present data indicate that the O-D change is not a discontinuous mutation effected by adaptation; but rather that it is a reversible phenomenon and is associated with characteristics which seem to be inherent in the virus particle.

It might be questioned whether the O phase virus was derived from D phase virus in the present experiments or whether the emergence of the O phase suspensions was the result of multiplication of a few O phase particles that had persisted. Obviously, that question cannot be settled with certainty because methods analogous to plating of bacteria are not available, by which growth from a single virus particle may be obtained. However, in the event that the reversion of D to O had been the result of growth from a few O particles, the change should have been a progressive replacement of the D by the O. But such was not the case. The emergence of the O phase was rather sudden and quite haphazard. The haphazard occurrence of the O form of virus agrees with the observation of Hirst (3) that the O form occurred in only a few embryos

out of many inoculated. The abrupt and unpredictable manner in which the O or D form appeared following inoculation of either form, strongly suggests that both are inherent characteristics of the virus particle,—perhaps of a genetic-like nature. In that respect, it is of interest that Francis and Moore (5) concluded that the pneumotropic-neurotropic variation of the WS strain also was a characteristic inherent in the virus particle.

Tropism seems to be of importance in the O-D phenomenon, but its exact rôle is not clear. The present data agree with those of Burnet and Bull (1) that allantoic passage rather uniformly yields allantoic fluid in which the D form dominates, whereas amniotic passage of infected embryo lung and trachea may yield amniotic fluid in which either the O or D form may dominate. The D form thus appears to be less fastidious than the O. However, the present experiments with *in vitro* cultures show that the O form does multiply in cells of the chorio-allantoic membrane.

The demonstration that allantoic fluids which under usual test conditions exhibit O form hemagglutination, may be made to exhibit hemagglutination of the D form merely by adjustment of the pH of the system is of interest from the viewpoint of the mechanism of virus hemagglutination. It does not alter the significance of the O-D variation as such. But, it does suggest that the failure of O form virus to agglutinate chicken erythrocytes is analogous to the failure of some strains of influenza A virus to agglutinate sheep erythrocytes (9). In both instances it appears that substances present in allantoic fluid render the test system unsuitable for hemagglutination; but when the systems are adjusted with phosphate-citric acid buffer, agglutination of erythrocytes readily occurs.

SUMMARY

Data are presented which enhance the idea that the influenza virus is an unstable agent. They indicate that the O-D type of variation is not a discontinuous mutation but rather is a reversible phenomenon. The O and the D forms of virus both appear to be inherent in the virus particle; the dominance of one or the other form seems to be subject to chance occurrences, but is influenced by the conditions under which the virus is propagated.

The capacity of the O form of virus to agglutinate guinea pig but not chicken erythrocytes is a relative, not an absolute phenomenon; allantoic fluids which exhibit clear-cut O form hemagglutination may be made to exhibit D form merely by addition of suitable buffer to the test system. That point is of importance from the viewpoint of the mechanism of influenza virus hemagglutination.

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PARENTERAL PLASMA PROTEIN MAINTAINS NITROGEN EQUILIBRIUM OVER LONG PERIODS

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The main thesis of this paper is the "dynamic equilibrium" between body protein and plasma protein. A steady state or balance exists between the body protein stores, protein wear and tear, and protein production. Protein production includes the plasma proteins which probably represent the largest fraction of new formed protein. Body protein reserve stores are largely intracellular. We believe the plasma proteins are the means of a fluid interchangé between reserve stores and the organ cells in which protein is produced, modified, and utilized in the body economy. The term "protein pool" suggests this fluid exchange within the body.

Earlier experiments in this laboratory showed that plasma protein as plasma as the sole source of protein given by vein could maintain nitrogen equilibrium for 2 to 4 weeks (3, 11, 13). The last report (3) showed evidence of some "intoxication" which developed in two of these experiments. An attempt to reproduce this "intoxication" in the experiments tabulated below was not successful, and we are inclined to explain the "intoxications" previously described as due to contaminated plasma or vitamin deficiency or both. The experiments given below are quite satisfactory and diet deficiencies were guarded against. The plasma was handled with more complete asepsis so we conclude that "intoxication" as described (3) is not an inevitable part of the experiment when large volumes of dog plasma are given parenterally.

In several experiments in other series at various intervals the dog's circulating plasma was fractionated and various elements estimated after the parenteral injection of plasma had been started—*e.g.*, fibrinogen, globulins, albumins using coagulation, chemical methods, and electrophoretic technique. No uniform deviation from the normal plasma protein pattern was observed. Depleted dogs have a tendency to show some increase in globulins at the expense of albumins. It seems to us that the behavior of *fibrinogen* is a very good illustration of the fact that long continued plasma given over months does not modify significantly the level of any plasma protein in the circulation. Fibrinogen is a very special protein used in the body for the production of blood clots. Yet obviously it is used in the body economy presumably within cells to supply special cell needs (other than coagulation) since otherwise the fibrinogen

would accumulate. This is true of all blood plasma proteins, the evidence being conclusive that all these various plasma proteins do serve the purpose of body cell protein replacement.

There have been differences of opinion (4–6) relative to the type of experiment here described. In certain experiments (6) there was evidence of primary nitrogen retention and subsequent nitrogen excess excretion. We believe this reaction was due to the citrate used to collect plasma and that the use of heparin will give the response tabulated below. Some writers have criticized our early experiments as being too short—that the plasma proteins were simply tucked away as inert protein in body tissues to be released in the after period. These objections are surely met by continued experiments of 2 to 3 months in which the dog was maintained in nitrogen and weight balance and in a state of lively well being all this time.

These experiments are not easy and call for meticulous technique, dogs conditioned to unusual food mixtures (very low protein diet), freedom from infection, and attention to all diet factors. Peritoneal absorption of plasma is rapid and in some instances less disturbing to the dog than intravenous injection. It obviates some rapid blood volume fluctuations.

Methods

Plasma was obtained from a colony of 10 large healthy donor dogs maintained on kitchen scraps to which a tablespoon of powdered lextron (Eli Lilly and Co.) was occasionally added. (Lextron is a liver extract plus iron.) Blood was drawn into sterile flasks containing sufficient heparin to prevent clotting. The blood was poured into sterile rubber-capped 100 cc. centrifuge tubes and spun at high speed for 20 to 30 minutes. The plasma was suctioned off into a sterile flask, and after thorough mixing an aliquot was taken for Kjeldahl analysis. The remainder was placed in a sterile gravity injection bottle and the plasma was administered immediately in a single intravenous or intraperitoneal injection each day. The time for administration varied from 10 to 20 minutes.

The two recipient dogs were adult healthy mongrel females under observation in the experimental colony for years. (See experimental histories below for details.) During the experiment the dogs were kept in galvanized iron metabolism cages in the laboratory under constant supervision, with access to water at all times. Before the initial dose of plasma was given in each experiment, the red cells of the recipient dog were cross-matched with the serum or plasma of each donor dog to exclude isoagglutinins and isohemolysins (18).

Blood for analysis was placed in graduated centrifuge tubes, using 1 cc. of a 1.4 per cent solution of sodium oxalate as an anticoagulant. This was centrifuged at high speed for 30 minutes and the total nitrogen in the plasma determined by macro Kjeldahl analysis using 1 cc. samples of plasma in triplicate. The usual factor 6.25 was used to convert to grams of protein. Because hematuria was encountered early in the first experiment, the procedure of catheterization at the end of each period was discontinued and the periods were terminated simply by changing collection bottles containing 5 cc. of toluene as a preservative every 48 hours. The total urinary nitrogen was determined by macro Kjeldahl analysis using 2 cc. aliquots in triplicate. Urea and ammonia nitrogen were determined simultaneously by aeration following digestion with urease using 2 cc. samples in duplicate. The amount of protein nitrogen in the urine was determined by precipitation of the protein in 10 cc. of urine by 90 cc.

of 5 per cent trichloracetic acid. In the first half of the first experiment, the filtrate was analyzed to determine the non-protein nitrogen content of the urine, and the urinary protein nitrogen arrived at by difference. Because of the possibility of magnification of small errors by this procedure, all subsequent determinations represent total nitrogen analyses of the urinary protein precipitated and collected on filter paper.

The dogs received only 50 gm. of glucose dissolved in 200 cc. water by stomach tube daily except during the periods when they received a very low protein diet as indicated in the tables.

The *very low protein diet* contained 0.012 per cent nitrogen and was made as follows: Sucrose 2600 gm., bone ash 78 gm., salt mixture 78 gm., liver powder, (Lilly B_1) 26 gm., powdered yeast (type 200-B) 26 gm. The above ingredients were thoroughly mixed, and then the following was added in portions, mixing thoroughly with each addition: Mazola oil 235 gm., cod liver oil 50 gm., lard (melted) 490 gm. Final mixing was done until the mass was homogenenous. The salt mixture was Wesson's modification of the Osborne-Mendel mixture (17).

Whenever the dogs received this low protein diet, 25 mg. of nicotinic acid and 200 mg. of choline were added daily as a vitamin supplement. The total N content of the casein used was determined accurately by macro Kjeldahl analysis (13.1 per cent). The figures in the tables represent the calculated nitrogen intake from all possible sources including casein, vitamins, and low protein diet. All sources of nitrogen are accounted in the Summary Table.

Special vitamin mixture—each 10 cc. daily dose contained: Vitamin A 5,000 U.S.P. units, thiamin hydrochloride 6.0 mg., riboflavin 6.0 mg., pyridoxine hydrochloride 5.0 mg., calcium pantothenate 5.0 mg., nicotinamide 50.0 mg., inisotol 200.0 mg., paraminobenzoic acid 50.0 mg., ascorbic acid 50.0 mg., 2-methyl-1,4-naphthoquinone 1.0 mg., vitamin D 500 U.S.P. units, rice polish concentrate 1.0 mg., linoleic acid 500.0 mg., choline chloride or citrate 400.0 mg., distilled natural tocopherols 50.0 mg. The emulsifying agent was pectin (supplied to this laboratory by Eli Lilly and Co.). Kjeldahl analysis showed this material to contain 74 mg. N in each daily dose. Whenever this mixture was added to the glucose solution for administration by stomach tube, the extra nitrogen appears in the tables under Diet, Total N

EXPERIMENTAL OBSERVATIONS

Tables 1 and 1-concluded give all the experimental data relating to the longest experiment with intravenous injections of normal dog plasma. Following a fasting period of 2 days, *dog 43-346* was given only 50 gm. of glucose by stomach tube daily for 10 days. This allowed the nitrogen equilibrium to reach a base line level. The urinary protein nitrogen in the first two periods represents a gross hematuria caused by catheterization which promptly disappeared when this procedure was discontinued.

The base line total urinary nitrogen (periods 2 and 3 of Table 1) is about 7 gm. per 4 days. During periods 4, 5, and 6 the total urinary nitrogen is below 6 gm. per 4 day period in spite of plasma protein parenterally (8.5 to 9.8 gm. per period). This represents a positive urinary nitrogen balance of 10.5 gm., due in part perhaps to a repletion of reserve protein stores. In period 9 we note a urinary nitrogen of 12.4 gm. but about 2 gm. belong to period 8 due to urinary carry over.

The non-protein diet replacing the sugar brings the urinary nitrogen back to its base line fasting level or below and there is a strong positive N balance. *Weight gain* is observed (periods 11 to 17). The urea and ammonia fraction of

the urinary nitrogen (Table 1) shows no unusual change and is compatible with a frugal metabolism of body protein. Some of the irregularities of the urinary nitrogen figures are due to residual urine because catheterization was contra-indicated.

The second interval (Table 1-concluded) of sugar by mouth and parenteral plasma (periods 18 to 27) does not show as complete nitrogen conservation for these 40 days as was observed in the first comparable interval (periods 4 to 10). There is a negative urinary nitrogen balance of 4.3 gm.—the weight loss is the same as in periods 4 to 10. During this second interval there is a continuous albuminuria (total 6.1 gm. urinary protein N). Undetermined nitrogen remains unchanged. These factors are probably related (urinary protein and slight negative urinary nitrogen balance), and explain in part the less efficient protein conservation; also the protein stores are probably repleted in the presence of the hyperproteinemia of 10 to 11 gm. per cent. When the parenteral plasma protein is replaced by casein by mouth the proteinuria promptly

TABLE 1

Plasma Parenterally Plus Sugar by Mouth

Dog 43-346

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
1			8.77	5.91	67.4	1.40	1.46		9.6	−8.77
2			7.09	5.18	73.1	1.15	0.36			−7.09
3*			3.62	2.81	77.4	0.81	0	8.17	8.6	−3.62
4		9.79	5.98	4.77	79.6	1.21	0			3.81
5		8.57	5.51	4.45	80.8	1.06	0	9.90		3.06
6		9.56	5.91	4.79	81.1	1.12	0	10.40	8.2	3.65
7		7.88	7.63	6.36	86.4	1.16	0.11			0.25
8		9.65	7.17	5.75	80.2	0.86	0.56		8.1	2.48
9		10.16	12.41	9.46	76.1	1.17	1.81			−2.25
10*		4.47	5.06	3.72	73.5	0.51	0.83	9.99	7.6	−0.59
Non-protein diet begins; sugar ends										
11	0.15	8.60	7.49	4.93	66.0	1.02	1.34			1.26
12	0.15	7.43	6.53	4.82	73.7	1.15	0.56	10.20	8.1	1.05
13	0.15	9.97	5.42	4.20	77.5	0.90	0.32	9.34	8.4	4.70
14	0.15	8.95	6.49	4.76	73.3	1.37	0.36	10.00		2.61
15	0.15	9.69	7.35	5.67	77.2	1.08	0.60		8.4	2.49
16	0.15	9.41	6.11	4.66	76.3	1.04	0.41	9.85	9.0	3.45
17*	0.08	4.21	4.11	3.29	80.0				8.9	0.25

* 2 day periods.

TABLE 1—*Concluded*

Plasma Injections Plus Sugar by Mouth
Proteinuria Disappears When Plasma Injections Cease

Dog 43-346

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
18		9.17	8.24	6.89	83.6	1.17	0.18	9.86	8.5	0.93
19		8.56	10.00	8.14	81.4	1.36	0.50	10.35		−1.44
20		9.43	7.98	7.03	88.1	0.67	0.28	10.54	8.3	1.45
21		8.50	10.05	8.18	81.4	1.18	0.69	10.67		−1.55
22		8.69	8.41	6.94	82.5	0.99	0.48	11.46	7.8	0.28
23		8.44	9.95	7.77	78.0	1.28	0.90	9.26		−1.51
24		8.27	8.06	6.71	83.2	0.88	0.47			0.21
25		8.52	8.34	6.75	81.0	0.79	0.80	11.00	7.4	0.18
26		8.34	10.65	8.19	76.8	1.50	1.16	11.24		−2.31
27		8.38	8.89	7.11	80.0	1.08	0.70			−0.51
Casein plus non-protein diet begin, plasma injections end										
28	6.00		6.66	5.16	77.5	1.20	0.30	8.22	7.9	−0.66
29	6.00		7.30	5.88	80.6	1.42	trace	7.32		−1.30
30	6.00		4.23	3.02	71.3	1.21	0	7.84	7.8	1.77

Total figures given under Experimental observations.

clears, indicating no renal injury, and the urinary nitrogen decreases. There is a suggestion here that the utilization of parenteral plasma protein has a ceiling beyond which the body cannot use as efficiently this available plasma protein. The renal threshold for plasma protein is probably a part of this limitation of internal protein metabolism.

The total *volume of plasma* injected was 19,173 cc. in 92 days, averaging 208 cc. per day. During periods 4, 5, 7, and 8, the plasma was given intraperitoneally without reaction. Slight diarrhea occurred on only one day, which did not duplicate the troublesome diarrhea reported in previous experiments in this laboratory (13).

The total amount of *nitrogen injected* in this experiment (Table 1, periods 4 to 27 inclusive) was 204.63 gm. averaging 2.22 gm. per day. The total urinary output of nitrogen during this 92 day period was 183.54 gm. averaging 2.0 gm. per day. If we assume 0.2 gm. N is lost daily in the feces, the dog would still be in positive nitrogen equilibrium for the over-all period. (See Summary Table.)

The proteinuria noted after the 14th day of plasma injections is a phenomenon to be discussed in detail in a subsequent paper. Suffice it to say

here that protein appears in the urine quite regularly in our dogs whenever the protein stores are repleted and the circulating plasma protein concentration is greater than 9.5 gm. per cent. Dogs may show some individual variations in the appearance and the severity of the proteinuria. This proteinuria disappeared quite promptly when the plasma injections were discontinued, indicating lack of kidney injury.

During the intervals when the entire caloric intake of the dog was represented by only plasma and glucose, she lost weight at a rate closely approximating the expected weight loss if the extra calories needed for the dog's daily nutrition came from her own body fat. During the second part of Table 1, periods 11 to 17, the dog received adequate calories in the form of a very low protein diet (145 gm. daily of the mixture described under Methods). A gain in weight resulted.

The other two experiments were run simultaneously and the pooled plasma from each day's bleeding of the donor dogs was divided between the recipient dogs.

Dog 43-346 (Table 2) received a total of 18,090 cc. of plasma in 76 days averaging 238 cc. per day. The total amount of nitrogen injected in this experiment was 177.66 gm. averaging 2.34 gm. N per day. The total urinary output of N was 153.62 gm. averaging 2.02 gm. N per day. By assuming a

TABLE 2

Plasma Intravenously; Sugar by Mouth

Dog 43-346

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm. per cent*	*kg.*	*gm.*
1		0	7.89	6.29	79.8	1.60	0	6.79	10.8	−7.89
2		0	9.15	7.46	81.6	1.69	0	6.47	10.4	−9.15
3		0	5.93	4.30	72.6	1.63	0		10.1	−5.93
4		0	5.01	4.41	88.1	1.10	0	6.17	9.8	−5.01
5		7.54	4.74	3.55	74.9	1.15	0	7.18	9.9	2.80
6		7.90	3.75.	2.76	73.7	0.99	0	8.70	9.9	4.15
7		6.49	4.87	3.84	78.8	1.03	0	9.27	9.8	1.62
8		7.54	6.02	4.76	79.0	1.26	0	9.07	9.6	1.52
9		7.24	6.17	5.01	81.2	1.16	Trace	9.57	9.6	1.08
10		8.01	3.74*	3.14	84.0	0.60	0	9.85	9.5	4.27
11		9.23	10.56	9.00	85.2	1.44	0.17	10.28	9.2	−1.33
12		9.56	9.62	7.78	80.9	1.39.	0.45	9.98	9.0	−0.06
13	0.3	9.61	8.94	7.51	84.0	1.11	0.26	10.03	9.0	0.97
14	0.3	10.07	9.90	7.17	72.4	1.25	0.48	10.55	8.9	0.47

* Vomiting.

TABLE 2—*Concluded*

Plasma Intraperitoneally; Sugar Plus Vitamins by Mouth

Dog 43-346

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
15	0.3	7.57*	9.82	6.53	66.5	0.89	2.40‡	10.22	8.7	−1.95
16	0.3	9.80	8.03	6.57	81.8	1.07	0.39	10.10	8.7	2.07
17	0.3	11.07	7.89	6.42	81.4	1.04	0.43	10.30	8.9	3.48
18	0.3	12.33	8.09	6.84	84.6	0.73	0.52	10.40	8.8	4.64
19	0.3	10.31	9.29	7.33	78.9	0.97	0.99	11.02	8.5	1.32
20	0.3	10.91	10.29	7.52	73.1	0.98	1.79	10.98	8.3	0.92
21	0.3	10.07	12.38	9.08	73.4	1.19	2.11	10.75	8.2	−2.01
22	0.3	10.98	10.86	7.75	71.4	0.85	2.26	10.90	8.0	0.42
23	0.3	11.53	9.66	6.71	69.5	1.27	1.68	10.78	7.8	2.17
	Plasma and sugar cease; casein plus non-protein diet begin									
24	8.00		10.37	7.68	74.1	1.28	1.41	10.10	8.0	−2.37
25	8.00		8.52	6.87	80.6	1.15	0.50	8.67	8.0	−0.52
26	8.00		8.25	7.00	84.6	1.13	0.12	7.38	7.8	−0.25

* Does not include 2.4 gm. injected into bladder.

‡ Plasma given by mistake into bladder.

total fecal nitrogen of 15.20 gm. for the same period, the dog again remained in nitrogen equilibrium. (See Summary Table.)

Dog 44-98 (Table 3) received 18,350 cc. of plasma averaging 241 cc. per day. The total nitrogen injected was 180.29 gm. averaging 2.37 gm. N per day. The total urinary output in this same period was 167.65 gm. N. If the fecal nitrogen is estimated at 15.2 gm., the total nitrogen output is 182.85 gm. Since this dog received 48.1 gm. of N from casein and vitamins during the last 6 periods of plasma injection, she was in strong positive nitrogen balance for the total period. (See Summary Table.)

Tables 2 and 3 give the results of *intravenous* injection and Tables 2- and 3-concluded show that *intraperitoneally* administered plasma was utilized equally well. After a 2 day fast, each dog was maintained for 16 days on 50 gm. of glucose daily by stomach tube. Dog 44-98 (Table 3) received a mixture of vitamins (see Methods) each day with the glucose. Dog 43-346 (Table 2) received no vitamins at the start of the experiment. During the 10th period, after 3 weeks of plasma intravenously, this dog began to vomit daily and to show signs of toxocity although no significant negative nitrogen balance was encountered. The experience of earlier workers (3) was not repeated—intoxication and marked loss of nitrogen.

Because dog 44-98 was simultaneously showing significant amounts of protein in her urine and seemed to be healtuy except for loss of weight, injectable vitamin B was added to the plasma and the special vitamin mixture was added to the daily glucose diet (dog 43-346) (see Experimental history, dog 43-346). The vomiting stopped almost immediately even though it seemed to be partly a conditioned reflex. From this point until period 18, Table 3, the dogs received identical treatment and appeared in excellent condition except for the obvious loss of weight.

During the next 6 periods dog 44-98 (Table 3-concluded, periods 18 to 23) received 200 mg. choline, 25 mg. nicotinic acid, and 140 gm. of the non-protein diet daily plus sufficient casein to give 2.0 gm. N per day in addition to the plasma. The dog gained weight on this regime and the amount of protein in the urine increased. Each dog had mild diarrhea on only 1 day during the intraperitoneal injections.

During periods 24 to 26 the proteinuria in each dog decreased rapidly and on the 12th day following the last plasma injection no trace of protein was found in the urine from either dog. There was also no evidence of wastage or escape of the parenterally administered protein in the form of excess urinary nitrogen during this after period.

At no time did the experimental dogs develop a significant diarrhea. Because no solid food was supplied except at the times indicated in the tables, the volume of feces was always small. Previous investigations in this laboratory

TABLE 3

Plasma Intravenously; Sugar Plus Vitamins by Mouth

Dog 44-98

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm. per cent*	*kg.*	*gm.*
1	0.3	0	4.95	3.65	73.7	1.30	0	6.70	9.0	−4.65
2	0.3	0	4.81	3.58	74.4	1.23	0	6.20	8.5	−4.51
3	0.3	0	3.68	2.53	68.8	1.15	0		8.3	−3.38
4	0.3	0	3.61	2.64	73.2	0.97	0	5.77	8.2	−3.31
5	0.3	7.40	3.46	2.36	68.2	1.10	0	6.88	8.2	4.24
6	0.3	8.15	3.22	2.10	65.2	1.12	0	8.59	8.1	4.23
7	0.3	6.71	4.84	3.71	76.6	1.33	0		8.2	2.17
8	0.3	7.88	5.39	4.18	77.6	1.21	0	8.70	8.0	2.79
9	0.3	7.46	5.80	4.18	72.1	1.62	0	9.40	7.9	1.96
10	0.3	8.53	6.02	4.65	77.3	0.52	0.85	9.82	7.9	2.81
11	0.3	9.47	7.72	4.82	65.0	0.84	1.76		7.7	2.35
12	0.3	9.27	8.12	5.58	68.7	1.17	1.37	9.79	7.6	1.45
13	0.3	9.64	7.73	5.82	75.3	0.50	1.41	10.19	7.4	2.21
14	0.3	10.20	8.43	5.79	68.7	0.79	1.85	10.11	7.4	2.07

TABLE 3—*Concluded*

Plasma Intraperitoneally; Casein Plus Non-Protein Diet

Dog 44-98

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
15	0.3	10.82	9.50	6.10	64.2	1.06	2.34	10.08	7.2	1.62
16	0.3	10.29	9.41	5.62	59.8	1.11	2.68	10.06	7.2	1.18
17	0.3	11.70	9.60	5.79	60.3	1.10	2.71	10.33	7.2	2.40
Sugar ends; casein plus non-protein diet begin										
18	8.00	10.13	11.28	6.66	59.0	1.17	3.45	10.25	7.3	6.85
19	8.00	11.46	12.83	7.89	61.5	1.33	3.61	10.46	7.5	6.63
20	8.00	10.28	12.20	7.20	59.0	1.25	3.75	10.21	7.6	6.08
21	8.00	9.46	13.48	8.95	66.4	0.99	3.54	9.64	7.8	3.98
22	8.00	10.30	14.45	9.50	65.7	1.78	3.17	9.86	7.9	3.85
23	8.00	10.59	14.47	9.40	65.0	1.31	3.76	10.04	8.0	4.12
Plasma ends; casein and non-protein diet continue										
24	8.00		12.43	9.04	72.8	1.60	1.79	9.13	7.9	−4.43
25	8.00		9.99	8.45	84.6	1.09	0.45	7.46	7.9	−1.99
26	8.00		7.66	6.48	84.6	1.08	0.10	6.72	7.8	0.34

(13) have shown that the fecal nitrogen is relatively constant under these basal conditions and varies from 0.1 to 0.2 gm. N per day. Therefore, fecal nitrogen was not determined in these experiments and the figure of 0.2 gm. N per day was assumed as a generous estimate in calculating the nitrogen balance for each dog.

The same remarkable conservation of injected plasma protein nitrogen is shown in *Table 2* as in Table 1 (periods 5 to 7). The total urinary N is below the minimum fasting level, giving a fairly large positive N balance of 8.57 gm. During periods 8 to 18 the total urinary N rises slowly but remains much below the intake of plasma N. Proteinuria begins and the urinary N rises slowly, in part owing to this protein N (periods 12 to 14). The plasma protein levels have now reached 10.0 gm. per cent. Conditions remain unchanged through periods 15 to 19. Positive urinary nitrogen balance is maintained. Proteinuria increases sharply in periods 20 to 23 although the plasma protein levels are unchanged. The undetermined N and the urea and ammonia N are little changed. Positive urinary N balance continues. During all this time, periods 5 to 23, there has been 2 kilos weight loss, with continuing sugar by mouth and plasma parenterally. When casein by mouth plus non-protein diet replaces the plasma intake there is no change in weight and no gain in N balance.

Table 3 shows some differences on comparison with Tables 1 and 2. We note

the extreme N conservation of 8.47 gm. in periods 5 and 6 where the minimum fasting level is above the levels of these first two periods of plasma administration. Periods 7 to 10 show strong positive urinary N balance (9.73 gm.) but the urinary N is rising a little. Periods 11 to 14 show a severe proteinuria but a continuing positive urinary N balance. Periods 15 to 17 show even more proteinuria, but the non-protein N and the urea-ammonia N show little change. During this long interval (periods 5 to 17) there is only 1 kilo weight loss—sugar by mouth and plasma parenterally. This dog develops a strong proteinuria at an earlier date, which goes much beyond the levels recorded in Table 2 though the plasma protein levels are if anything less. Recovery from the proteinuria is equally prompt in both dogs when plasma injection is discontinued.

When casein plus non-protein diet replaces the sugar by mouth and plasma continues we note that the proteinuria increases but not the plasma protein levels. There is a strong positive N balance, the urea-ammonia fraction increases, and there is 0.8 kilo weight gain. In period 24 it could be claimed that there is some loss of stored protein but the amount is insignificant.

SUMMARY TABLE

(Refer also to Summary Table in following paper.)

Dog No.	Periods	Total days	Nitrogen intake			Nitrogen excreted in urine		Protein nitrogen in urine	Estimated fecal nitrogen	Positive nitrogen balance
			From plasma		From diet					
			Daily average	Total	Total	Daily average	Total			
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
43-346	4–27	92	2.22	204	1.00	2.00	184	13.36	18.4	2.6
43-346	5–23	76	2.34	178	2.96	2.02	154	13.96	15.2	18.8
44-98	5–17	52	2.27	118	3.95	1.71	89	14.97	10.4	22.6
44-98	18–23	24	2.58	62	48.1	3.29	79	21.28	4.8	26.3

Experimental History—Tables 1 and 1–concluded.

Dog 43-346, normal adult female mongrel used previously for hemoglobin studies. Dog was given regular kennel ration (kitchen scraps) for several months prior to the experiment. Traumatic hematuria encountered at the end of the first period precluded further catheterization. Urine from each period was tested for protein with heat and dilute acetic acid. As soon as proteinuria appeared (period 7), the amount of protein nitrogen was determined quantitatively. Diarrhea was present only 3 times and vomiting only once during the entire experiment. The dog gained weight whenever adequate calories were supplied and was in excellent general condition at the end of the experiment.

Experimental History—Tables 2 and 2–concluded.

Dog 43-346 (Table 1) was given a rest period of 4½ months on a diet of kitchen scraps. Repeated qualitative tests during this period revealed no trace of protein in the urine. During the 10th period (Table 2) the dog vomited copious amounts of watery fluid subsequent to the tube feeding. This probably caused some dehydration and oliguria. Because catheterization was contraindicated, there must have been some carry over of urine into period 11 to

account for the abnormally low total urinary nitrogen in period 10 and the abnormally high values in period 11.

Following this the dog began to salivate and vomit after every tube feeding. She apparently became conditioned to the point where vomiting would occur whenever the cage door was opened. Coprophagy was noted and during the first half of period 12 she again had oliguria. Tube feeding of sugar was stopped and crystalline vitamin B complex (solu-B, Upjohn) was added to the plasma for 6 successive days. Vomiting ceased and the animal lost her appearance of toxicity. When the 50 gm. of glucose by stomach tube was resumed, 10 cc. of the special vitamin mixture was added daily and no further vomiting or symptoms of toxicity were noted during the remainder of the experiment. On the last day of period 15, the entire dose of plasma was inadvertently injected into the dog's bladder without ill effect. This is reflected in the high figure for protein in the urine for this period.

Again, because of incomplete emptying of the bladder at the end of the last period of plasma administration (period 23), the urinary nitrogen excretion during the next period, when the dog received casein and low protein diet, was high.

Dog was frisky and healthy at the end of experiment, showing only evidence of weight loss.

Experimental History—Tables 3 and 3-concluded.

Dog 44-98, an adult mongrel female previously used for hemoglobin studies. Fed for several months prior to this experiment on kitchen scraps. She was given 10 cc. of special vitamin mixture daily with the 50 gm. glucose by stomach tube; no protein in the urine during 2 day preliminary fast. Qualitative tests revealed no proteinuria until period 10. No vomiting or diarrhea was noted at any time. The dog remained in excellent health except for loss of weight throughout the period of 52 days while receiving plasma as the sole source of nitrogen (plus 0.074 gm. from the vitamin mixture daily). From period 18 through 23 she received adequate calories and nitrogen from the casein and non-protein diet in addition to the plasma intraperitoneally. Weight gain was only moderate and fell off slightly when the plasma was stopped. Dog in excellent general condition at end of experiment.

DISCUSSION

The plasma proteins introduced into the dogs evidently contributed to the various body tissues and organs and participated in the normal maintenance and body metabolism. Unlike protein by mouth there was no loss of nitrogen (12) and no evidence of any profound break down of the introduced plasma protein, but obviously this plasma protein must have been modified in some way to replace the worn out cell and tissue proteins. All these questions are capable of experimental approach and a beginning has been made by various workers (7, 10, 14). It will be of much interest to follow the distribution of the introduced proteins, and this can be done with labeled amino acids built into the plasma protein molecules.

Using heavy nitrogen (N^{15}) to label lysine (8) it was possible to produce in the dog labeled plasma proteins which in turn were given intravenously to a a normal dog. This labeled plasma protein did not long remain in the circulation,—about 50 per cent disappeared within 24 hours,—but the disappearance did not subsequently continue at this rapid rate. It was not possible to determine the concentration of the lysine proteins in various organs because of high dilutions and limitations of heavy nitrogen analysis. Possibly radio-carbon-

labeled lysine may enable us to study the distribution of this amino acid in the body. This work is in progress.

Plasma volumes change readily under the above experimental conditions but the capacity of adjustment is very great (2, 9, 15, 16). The hematocrit values fall during intravenous injection of plasma but the fluid and proteins of the plasma promptly come to a balance and the protein concentrations are not measurably changed when large amounts of plasma containing 6 gm. per cent plasma protein are given to a dog with hyperproteinemia (10 per cent protein). Samples taken at 3, 35, and 240 minutes showed remarkably close agreement in protein concentration. Observations on human beings (1) indicate that following plasma injections fluid may leave the blood stream at rates of 100 cc. per minute and plasma protein may be removed at rates of 4 to 6 gm. per minute.

As one studies the three long experiments described above a pattern emerges. Following a fast of 12 to 16 days the *urinary N reaches a minimum level.* The protein stores of the body must be definitely depleted and protein wear and tear and turnover are minimal. Now whole plasma given parenterally with sugar by mouth presents an extraordinary conservation of N and we note the urinary N *falls below the fasting level.* The urea-ammonia fraction shows no significant change. This remarkable N conservation continues for 8 to 12 days and presumably there is considerable repletion of body protein stores or reserves. The protein wear and tear and wastage is minimal. After this interval the urinary N rises a little, but nitrogen equilibrium is maintained. Proteinuria begins in 12 to 24 days and about this time the circulating plasma protein levels reach 9.5 to 10.5 gm. per cent. The degree of the proteinuria varies widely from 0.2 to 1.8 gm. in some dogs to 0.8 to 3.0 gm. of protein nitrogen per 4 day period. The animal is still in nitrogen equilibrium or positive balance, although the dog loses the urinary protein which does not enter into the internal body metabolism. There is no significant loss of urinary nitrogen when the plasma protein injections cease.

It seems probable that after the body protein reserves are repleted the dog can use only about so much plasma protein. Beyond this point hyperproteinemia develops (10 gm. per cent) and a good deal of the introduced protein will escape. The urinary N rises somewhat, but a positive N urinary balance is usually maintained. Given necessary carbohydrate, fat, salts, and vitamins by mouth with the parenteral plasma protein we note that weight equilibrium and normal health can be maintained for 3 months and probably very much longer. It is suggested that there is a ceiling for the use of introduced plasma protein when the depleted protein reserves are restored. This ceiling is high enough to permit maintenance of body nitrogen balance.

SUMMARY

Given adequate amounts of homologous plasma intravenously or intraperitoneally a protein-fasting dog can be maintained in nitrogen equilibrium for

several months, indicating efficient utilization of all plasma proteins in body metabolism. There is no accumulation of any specific plasma protein in the circulation, indicating that even highly specialized globulins (fibrinogen and others) are capable of participation in the general protein turnover and metabolism within the body. This is a fluid exchange or a *dynamic equilibrium* in protein metabolism. This exchange takes place without significant loss of nitrogen.

Body weight is maintained when adequate calories are supplied (very low protein diet) during the plasma injection periods.

No periods of unexplained intoxication were noted in the long experiments described. Health and activity were quite normal.

Continued hyperproteinemia with repletion of body protein stores and plasma protein levels of 9.5 to 10.0 per cent after 15 to 25 days produced proteinuria. A renal threshold for blood plasma proteins is suggested.

These experiments suggest that after protein reserves are repleted there is a limit or ceiling for the use of the introduced plasma protein, but this limit permits a nitrogen balance to be maintained. This limitation to the use of introduced plasma protein is closely related to the proteinuria.

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PROTEINURIA RELATED TO HYPERPROTEINEMIA IN DOGS FOLLOWING PLASMA GIVEN PARENTERALLY

A Renal Threshold for Plasma Proteins

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The evidence given below is adequate to establish a *renal threshold for plasma proteins.* Whatever the mechanism may be, there is a lag between the start of the plasma injections and the appearance of the proteinuria—a range of 4 to 26 days. We have not yet established how early the proteinuria may appear when very large doses of parenteral plasma are used nor have we made an effort to drive the hyperproteinemia above levels of 11.5 gm. per cent. The level of plasma protein concentration is probably most important in establishing a proteinuria—in normal dogs the values are 9.6 to 10.4 gm. per cent and probably individual variations are greater. Repeat experiments on the same dogs give practically identical values—see Summary Table—dogs 43-346 and 43-141. Prompt disappearance of the proteinuria, normal gross and histological findings in the kidney at autopsy follow discontinuance of plasma injections.

The literature is full of interesting papers dealing with the permeability of the glomerular tuft to substances other than electrolytes—proteins in particular. We do not propose a comprehensive review of this valuable material most of which has been recently discussed by Bing (4). The belief that the glomerular tuft in its normal state is permeable to proteins was slow in gaining acceptance. When the claim was made that the glomerular tuft was permeable to certain proteins, it was often argued that the glomeruli were abnormal or that there must be crevices or stomata between the various cells lining the capillaries and the epithelium covering the tuft.

The preceding paper dealing with the dynamic equilibrium of proteins—an ebb and flow between the plasma proteins and cell proteins—gives at least a suggestion relating to the proteinuria observed. When albumins or globulins are formed rapidly within a *liver cell* because of body demand, the proteins presumably accumulate within the cell until there is an outflow—due to an increased density of intracellular protein or other factors. Conversely in protein fasting when parenteral plasma is given, these liver cells (and others) permit a protein inflow—perhaps due in part to a decreased density of intracellular protein.

We know of no evidence that there are any stomata involved in these ex-

changes by the liver cell or muscle cell so why argue that there must be stomata or some permanent structural change to account for a passage of protein through normal cells just because they happen to be in the glomerular tuft? With increased concentration of proteins in the plasma these proteins in large amounts do pass the glomerular tuft—not only albumin but several varieties of the globulins. Furthermore this passage involves capillaries as well as the epithelium covering the glomerular tuft. When plasma is given intraperitoneally, it appears promptly in the circulation (passage of two cell barriers); and when it supplies the protein needs of the body, it must pass two more barriers (capillaries and cell membrane—muscle or liver cell for example). It seems obvious that plasma proteins within the body must pass cell barriers with ease. It is accepted that body cell membranes or barriers contain proteins and without doubt some enzymes (proteins) are included in the barrier. It has been suggested (19) that these enzymes may participate in the easy passage of albumins and globulins through cell membranes.

Well known publications support the hypothesis that protein molecules can pass the normal glomerular tuft (1, 8–10, 14–18). The problem of orthostatic albuminuria (20) belongs in this discussion. Hyperglobulinemia is discussed by Bing (5) in observed human cases with particular reference to multiple myeloma. Our dogs show a preponderance of globulins at times, especially when large amounts of albumin appear in the urine—increased sedimentation rate was obvious in these dogs and was recorded by Bing in human beings. Some workers explain the passage of proteins through the glomerular filter as purely a physical phenomenon (2). Bell (3) claims that the capillaries of the glomerulus are unlike those elsewhere in the body and the endothelial lining is not continuous. Monke and Yuile (13) discuss the size of the pores in the glomerular tuft as related to hemoglobinuria. Blackman *et al.* (6) discuss the pathogenesis of Bright's disease and emphasize that high urinary globulin concentration gives a bad prognosis in these patients.

EXPERIMENTAL OBSERVATIONS

The experiments in this paper are similar to those in the preceding paper and are to be examined as a single group; the methods are identical. Reference to the Summary Table below will give total figures for all experiments in these two papers.

Tables 4 and 5 should be examined together since the observations were made on the same dog, a rest period of 12 months on a liberal mixed diet intervening. Table 4 is the first experiment of the whole series and was pushed along week by week with the hope of producing "intoxication" with or without loss of nitrogen. Proteinuria was observed in period 6, Table 4, and increased steadily while the plasma injections continued, but vanished within 2 days after plasma injections ceased. When casein by mouth in relatively equivalent amounts replaces the

plasma by vein we note a frugal use of this protein, a continued positive nitrogen balance, and slight weight gain.

Table 5 is a more complete experiment on the same dog (Table 4), after a rest interval of 12 months. The dog in Table 5 weighs 1.7 kg. more and requires a longer period of fasting to settle down to a base line of urinary nitrogen output. About 2.5 times the amount of plasma protein was given at the start as compared with Table 4, and the amount of nitrogen *conservation* is spectacular in periods 5 and 6, Table 5—a total of 25 gm. nitrogen—presumably a repletion of protein reserves. With these larger injections of plasma the proteinuria appears earlier and on the 8th day the urine "boiled solid," the blood plasma protein levels standing at 9.7 gm. per cent. Because of the large amounts of protein in the urine the urea and ammonia fraction is a better index of nitrogen metabolism and shows insignificant changes. A positive nitrogen balance continues. Intraperitoneal injections are then used. Weight loss is very slow and the dog is in excellent condition. Proteinuria cleared 4 days after cessation of plasma injections.

Evidently larger injections of plasma cause an earlier appearance of proteinuria and a higher output of protein in the urine, representing in this dog 24 per cent of the protein injected (Table 5). The levels of blood plasma protein concentration are not strikingly different in Tables 4 and 5. The highest output of protein in the urine is noted in periods 15 to 17 (Table 5) with plasma protein concentration of 10.4 to 11.3 gm. per cent. When the plasma injections cease there is no notable escape of stored nitrogen (periods 18 and 19, Table 5).

Electrophoretic Studies (Dr. Eric Alling)

In general the *urinary protein* concentration ranges as high as 10 to 15 per cent of the plasma protein concentration but the albumin fraction makes up 60 to 75 per cent of the total urinary protein and fibrinogen does not appear in recognizable form in the urine. The other globulins, however, are well represented in the urine and in general follow the percentage representation in the plasma.

Electrophoretic Pattern—Period 7, Table 4

	Plasma	Urine
	gm per cent	*gm per cent*
Albumin	4 08	0.6
Globulins—alpha 1	*	0.06
Globulins—alpha 2	*	0.05
Globulins—beta and gamma	3 2	0.13
Fibrinogen		0
Total		0.84
Kjeldahl—total	9 95	0.64

* Obscured by opacities of unknown origin.

TABLE 4

Plasma Intravenously; Sugar by Mouth

Dog 43-141, adult mongrel hound.

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm. per cent*	*kg.*	*gm.*
1			6.11	4.67	76.3	1.44			11.3	−6.11
2			3.12	2.28	73.0	1.12		6.20		−3.12
3		7.08	5.10	3.79	74.3	1.31			10.0	1.98
4		7.63	4.37	3.15	72.1	1.22		9.44	9.8	3.26
5		9.58	5.83	4.57	78.3	1.29			9.8	3.75
6		10.00	7.60	5.38	70.8	1.25	0.97		9.7	2.40
7		9.84	8.31	5.65	68.0	0.83	1.83	9.95		1.53
8		9.83	7.99	5.23	65.5	1.03	1.73		9.4	1.84
9		10.91	10.19	6.56	64.4	1.46	2.17	11.50		0.72
			Sugar stopped. Non-protein diet started							
10	0.16	10.30	9.80	5.72	58.6		3.75	9.77	9.4	0.66
			Plasma injections stopped. Casein added to diet							
11	8.00		7.36	4.68	63.7	1.73	1.15	7.64	9.6	1.14
12	8.00		6.85	5.40	78.8	1.45	0	7.97	9.8	1.65
13	8.00		6.35	4.93	77.7	1.42	0			2.15
14	8.00		5.56	4.08	73.4	1.58	0	8.05		2.94
15	8.00		5.27	4.08	77.5	1.19	0	7.08	10.1	3.23

In period 9, Table 4 (Tiselius), plasma proteins—albumin—4.26, alpha 1—0.32, alpha 2, 3, and 4—2.39, beta, gamma, and fibrinogen—4.13. Total—11.1 gm. per cent, and Kjeldhal analysis, total—11.5 gm. per cent.

Further studies of the plasma and urinary proteins by the method of Tiselius were carried out in Table 5, period 14.

	Plasma	Urine
	gm. per cent	*gm. per cent*
Albumin	3.58	1.02
Globulins—alpha 1	1.82	0.10
Globulins—alpha 2	1.65	0.07
Globulins—beta and gamma	2.75	0.24
Fibrinogen	1.31	0
Total	11.11	1.43
Kjeldahl—total	10.79	1.43

TABLE 5

Plasma Parenterally; Sugar and Vitamins by Mouth

Dog 43-141, Experiment 2.

Period No. 4 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
1	0.3		8.27	6.81	82.4	1.46		5.94	13.0	−7.97
2	0.3		7.18	5.97	83.1	1.21		5.77	12.6	−6.88
3	0.3		5.96	4.70	79.0	1.26		5.56	12.2	−5.66
4	0.3		5.16	3.98	77.2	1.18		5.29	12.0	−4.86
5	0.3	18.42	4.49	3.19	71.0	1.10		8.05	12.2	14.23
6	0.3	19.97	9.26	5.74	62.0	1.24	2.18	9.70	12.3	11.01
7	0.3	16.46	15.15	8.25	54.5	1.52	5.38	9.93	12.2	1.61
8	0.3	12.89	12.53	7.10	56.6	1.29	4.14	9.79	11.9	0.66
9	0.3	13.16	12.41	7.65	61.6	1.42	3.34	9.83	11.7	1.05
10	0.3	12.49	11.30	7.09	62.8	1.28	2.93	9.71	11.5	1.49
11	0.3	12.43	10.10	5.90	58.4	1.48	2.12	9.33	11.5	2.63
			Intraperitoneal injections only							
12	0.3	11.82	8.98	5.96	66.4	0.87	2.15	9.81	11.4	3.14
13	0.3	12.99	9.67	6.82	70.6	1.21	2.64	9.55	11.4	3.62
14	0.3	13.77	11.02	6.16	55.9	1.62	3.24	10.42	11.4	3.05
15	0.3	14.35	13.32	7.11	53.4	1.51	4.70	11.13	11.4	1.33
			Intravenous injections only							
16	0.3	15.61	16.03	8.76	54.6	1.54	5.73	11.32	10.9	−0.12
17	0.3	15.98	14.65	7.63	52.1	1.71	5.51	10.67	10.7	1.63
			Plasma stopped. Sugar and vitamins by mouth continued							
18	0.3		8.09	5.64	69.8	1.39	1.06	8.04	10.4	−7.79
19	0.3		6.31	5.30	84.0	1.01	0	7.32	10.0	−6.01

Again in period 17, Table 5—

	Plasma	Urine
	gm. per cent	gm. per cent
Albumin	2.90	1.03
Globulins—alpha 1	2.45	0.12
Globulins—alpha 2	1.65	0.09
Globulins—beta and gamma	2.50	0.33
Fibrinogen	1.30	0
Total	10.80	1.57
Kjeldahl—total	10.70	1.67

Again in period 18, Table 5, about 102 hours after the last injection of plasma the electrophoretic pattern of the plasma read: Albumin—2.11 gm. per cent, alpha 1 and 2—1.74 gm. per cent, alpha 3—1.86 gm. per cent, beta—0.92 gm. per cent, gamma—0.94 gm. per cent, fibrinogen—0.75 gm. per cent, with a total of 8.32 gm. per cent.

The relatively low levels of albumin in the circulating plasma are presumably related in part at least to the more rapid escape of the smaller albumin molecule through the kidney.

TABLE 5-*a*

Period 10 in Table 5

Protein Excreted at Relatively Constant Rate

Time	Urine volume	Volume* plasma injected	Total nitrogen injected	Urinary protein nitrogen		Blood plasma concentration
				Expected	Observed	
	cc. per 12 hrs.	*cc. per 24 hrs.*	*gm. per 24 hrs.*	*gm. per 12 hrs.*	*gm.*	*gm. per cent*
9 a.m.–9 p.m.	550	310	3.17	0.392	0.334	
9 p.m.–9 a.m.	450			0.321	0.362	9.37
9 a.m.–9 p.m.	800	272	2.75	0.570	0.486	10.00
9 p.m.–9 a.m.	275			0.198	0.299	9.86
Total 48 hrs.....	2075	582	5.92	1.481	1.481	
9 a.m.–9 p.m.	690	355	3.56	0.495	0.493	9.59
9 p.m.–9 a.m.	315			0.226	0.294	9.71
9 a.m.–9 p.m.	700	302	3.01	0.502	0.416	9.70
9 p.m.–9 a.m.	300			0.217	*0.295	9.77
Total 48 hrs.....	2005	657	6.57	1.44	1.44	

* Plasma injected at 11 a.m.

Table 5-*a* shows a breakdown of period 10 in Table 5. The rate of urine excretion is not constant, and more urine is excreted during the day, but there is a continuous escape of protein in the urine both night and day. Evidently there is not an immediate outpouring of protein through the kidney, following the injection of whole plasma during the forenoon. The evidence favors a relatively constant excretion of proteins by the kidney—see Experimental history, Table 5-*a*.

Tables 6 and 7 should be considered together as the experiments were similar and both animals were *bile fistula* dogs. Duration of both experiments was short—15 and 16 days but the daily injection of plasma was large. Note that the periods are 48 hours in both experiments as compared with 4 day periods in Tables 4 and 5. Bile fistula dogs do not tolerate fasting and these periods were only 2 and 3 days.

Protein reserves are low in these dogs, but the fasting levels were probably not reached before the plasma injections were begun. Proteinuria in traces appeared in 48 hours (Table 6) and on the 4th day (Table 7). Both dogs show a positive nitrogen balance and body weight was maintained. The dog of Table 6 shows definite proteinuria at a plasma protein level of 8.7 gm. per cent but the other bile fistula dog (Table 7) shows proteinuria at a plasma protein level of 10.4 gm. per cent and this level is continuously high while the proteinuria continues (Table 7, periods 6 to 9). Evidently individual variations and not the bile fistula were responsible. Both dogs were autopsied subsequently and showed no significant renal abnormalities.

Table 8 gives an experiment which was started without knowledge that the apparently normal dog was suffering from a mild cystitis and pyelonephritis which became quite active during the later periods 12 to 15. This inflammation explains the strong negative nitrogen balance in periods 13 to 15. Autopsy some 3 months later revealed the extent of this inflammation. A long fasting period (30 days) precedes the plasma injections in period 8, Table 8, to determine whether the onset of the proteinuria would be earlier or more severe in this depleted state. There were traces of proteinuria (the significance not appreciated) before the plasma injections were started, but definite proteinuria began on the 8th day. The degree of proteinuria is not beyond the expected range except in periods 13 to 15 when the infection of the renal pelvis and bladder was causing obvious intoxication. The cause of this inflammation was a series of experiments on the animal some months before that of Table 8, in which daily catheterization was employed to terminate urinary collections. One notes considerable protein utilization and nitrogen retention in periods 8 to 11. The experiment indicates that a long fast does not of itself modify the reaction to plasma injections—also the fact that an infection may disturb the usual picture observed in the normal dogs (compare Tables 1 to 5 in Summary Table).

Experimental History—Table 4.

Dog 43-141, adult female mongrel hound; was given regular kennel ration for several months prior to this experiment. Two days of fasting were followed by 8 days of feeding 50 gm. glucose by stomach tube daily. Each period was terminated by catheterization. Plasma protein determinations were done on blood samples drawn immediately prior to the time of plasma injection—about 11 a.m. Plasma administered intraperitoneally in periods 3 and 4 and intravenously thereafter. During periods 9 and 10 there was some vomiting and slight diarrhea. This ceased as soon as the plasma injections were discontinued, and no significant intoxication was noted at any time. 150 gm. of low protein diet plus 200 mg. choline and 25 mg. nicotinic acid was given daily during periods 10 to 15. Casein (15 gm. daily) was supplied during the after period so that the total N in diet was 2.0 gm. per day.

Experimental History—Table 5.

Dog 43-141 (Table 4), after a rest period of almost a year on a diet of kitchen scraps, was fasted for 4 days and then given 50 gm. of glucose and 10 cc. of the special vitamin mixture by stomach tube daily for 16 days. The dog lost almost 2 kg. during these 3 weeks but seemed otherwise active and healthy. Large volumes of homologous plasma (357 to 548 cc.) were

given in single doses half intravenously and half intraperitoneally for the first 10 days of plasma administration. During periods 8 and 9 and 12 to 15 the plasma was given intraperitoneally only. At all other times the plasma was injected intravenously. Diarrhea was noted only twice (periods 14 and 19). The dog remained in excellent general condition throughout the experiment except for the expected loss of weight. Plasma was the sole source of nitrogen throughout the experiment except for the 0.074 gm. N present in the daily dose of vitamins.

Experimental History—Table 5-*a*.

Dog 43-141. Period 10, Table 5, was divided into 12 hour intervals by catheterizing the dog and washing out the bladder with normal saline. No difficulty was encountered. An at-

TABLE 6

Plasma Intravenously; Low Protein Diet and Vitamins by Mouth

Dog 46-79, bile fistula.

Period No. 2 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
1	0.24		4.60	3.37	73.2	1.23		5.83	11.0	−4.36
2*	0.12		2.96	2.22	75.0	0.74		6.65	10.7	−2.84
3	0.24	6.95	3.15	2.45	77.8	0.70	Trace	7.84	10.8	4.04
4	0.24	7.34	3.78	2.36	62.4	0.86	0.56	8.72	10.9	3.80
5	0.24	7.29	4.39	2.31	52.6	0.87	1.21	9.45	10.9	3.14
6	0.24	7.85	5.72	2.28	39.9	1.18	2.26	10.25	10.9	2.37
7	0.24	7.42	6.48	3.12	48.2	1.12	2.24	9.14	10.7	1.18
8	0.24	7.36	6.35	2.68	42.2	1.20	2.47	9.45	10.9	1.25
9	0.24	7.54	5.46	2.56	45.3	1.00	2.08	9.95	10.7	2.32
10*	0.12	3.85	2.95	1.23	41.7	0.69	1.03	9.80	10.7	1.02
11	0.15		3.79	1.90	50.0	1.06	0.83	9.26	10.1	−3.64
12	0.15						0.06	8.44	10.2	
13	0.15		4.62	3.29	71.1	1.33	Trace	8.47	9.9	−4.47
14	0.15		4.45	3.45	77.5	1.00		8.72	9.3	−4.30
15	0.15		4.32	3.37	78.0	0.95		8.34	9.1	−4.17
16	0.15		2.79	2.00	71.8	0.79		8.03	9.2	−2.54

*1 day period.

tempt was made to produce a constant urine volume without success. The night output was always less than the daytime volumes. One column of the table gives the values of urinary protein nitrogen calculated as follows:

$$\text{Expected urinary nitrogen in 12 hour period} = \frac{\text{Urine volume in 12 hour period}}{\text{Total urine volume in 48 hours}} \times \text{Total urinary nitrogen in 48 hours}$$

Experimental History—Table 6.

Dog 46-79, adult female mongrel hound with bile fistula, in the course of bile salt metabolism studies to be reported later by Dr. William B. Hawkins *et al.*, was given kennel diet plus 50 to 100 cc. of her own bile by mouth daily for 50 days prior to the start of this ex-

periment. The dog received 200 gm. of low protein diet plus 10 cc. of special vitamin mixture daily for 4 days prior to intravenous administration of plasma, and this diet was continued throughout the period of plasma injections and for 2 days after plasma was stopped. The dog evidenced some allergic reaction on the 1st and 3rd days of plasma administration, in the form of facial edema and transient flushing of skin with itching. This cleared spontaneously. Vomiting occurred following injection during periods 5 and 6, probably owing to large volumes of plasma (374 to 385 cc.) given in a short time (10 to 15 minutes). Slight jaundice appeared during periods 9 and 10 and continued for several days. The dog received only 50 gm. glucose plus 10 cc. of the vitamin mixture by stomach tube daily during periods 12 to 16.

Further experiments not related to this paper were finished and about 6 weeks after period 16 the dog was killed with ether. The kidneys in gross and in histological sections showed no significant abnormalities. The 2 weeks' proteinuria left no mark on these organs.

TABLE 7

Plasma Intravenously; Low Protein Diet and Vitamins by Mouth

Dog 46-9, bile fistula.

Period No. 2 days	Diet Total N	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
			Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
1	0.24		2.62	1.89	72.3	0.73		6.15	8.8	−2.38
2	0.24	7.47	2.59	1.80	69.4	0.79		8.04	9.0	5.12
3	0.24	7.24	3.00	2.05	68.6	0.95		9.34	9.1	4.48
4	0.24	7.33	3.20	2.39	74.6	0.81	Trace	9.90	9.1	4.37
5	0.24	7.56	3.54	2.23	63.0	0.70	0.61	10.37	9.1	4.26
6	0.24	7.30	6.89	3.67	53.3	1.11	2.11	10.34	9.1	0.65
7	0.24	7.23	6.55	3.20	48.9	0.77	2.58	10.47	9.0	0.92
8	0.24	7.46	5.71	2.83	49.3	0.88	2.00	10.48	9.0	1.99
9	0.24	6.85	3.69	1.80	48.9	0.71	1.18	10.44	8.9	3.40
10	0.24		4.58	3.01	65.8	0.80	0.77	9.53	8.9	−4.34
11	0.24		2.73	1.81	66.1	0.92	Trace	8.20	8.6	−2.49
12	0.24		2.30	1.72	80.0	0.58		7.83	8.4	−2.06

Experimental History—Table7.

Dog 46-9, adult female mongrel with bile fistula, in the course of other experiments to be reported later by Dr. W. B. Hawkins *et al.*, was given kennel diet plus 100 cc. of her own bile daily for 25 days prior to the start of this experiment. The dog received 200 gm. of low protein diet plus 10 cc. of vitamin mixture, 200 mg. choline, and 25 mg. nicotinic acid for 7 days prior to intravenous administration of plasma, and this diet was continued throughout the period of plasma injections and for 6 days after the plasma was stopped. Facial edema, flushing and itching of the skin occurred once in period 6 and vomiting was noted once in period 9. Otherwise the dog was in excellent health and lost practically no weight during the 16 days of plasma injection. The proteinuria disappeared completely after 2 days on kennel diet. The dog was used for other experiments and was sacrificed several months later. No gross or histologic abnormalities were noted in the kidneys.

Experimental History—Table 8.

Dog 43-290, adult female mongrel bull, used previously for various experiments. The dog

was given regular kennel ration for several months prior to this experiment and was somewhat overweight. Two days of fasting was followed by 28 days of feeding 50 gm. of glucose by stomach tube daily. Faint traces of protein were detected in the urine during this starvation period. Catheterization was not employed in this experiment but had been used as routine in previous experiments. Heavier traces of protein appeared in the urine during the first period on plasma injections. The dog rapidly became toxic and began vomiting after the second period of plasma injections. Small ulcers appeared on the oral mucous membranes during period 11 (Table 8) and diarrhea became frequent. Vomiting became a conditioned reflex

TABLE 8

Plasma Intravenously; Sugar by Mouth

Dog 43-290, cystitis and pyelonephritis.

Period No. 4 days	Plasma injected Total N	Urinary nitrogen					Blood plasma concentration	Weight	Urinary N balance
		Total	Urea plus ammonia		Undetermined	Urinary protein			
	gm.	gm.	gm.	per cent	gm.	gm.	gm. per cent	kg.	gm.
1							7.35	14.8	
2						Trace			
3									
4						Trace		13.2	
5									
6		4.42	3.17	71.8	1.25			12.5	−4.42
7		5.08	4.02	79.2	1.06		6.38	12.1	−5.08
8	8.32	4.44	3.30	74.4	1.14	Trace			3.88
9	8.50	4.38	3.28	74.9	1.10	Trace	9.54	11.8	4.12
10	8.91	6.79	4.98	74.0	0.81	1.00	9.83		2.12
11	8.58	5.27	3.47	65.6	0.65	1.15			3.31
12	9.49	10.00	5.46	55.0	1.95	2.59	9.55	11.6	−0.51
Sugar discontinued. Dog toxic—vomiting									
13	9.35	16.00	9.57	59.4	1.71	4.72		10.9	−6.65
14	9.48	24.86	16.05	64.6	2.83	5.98	10.04	10.3	−15.38
15*	5.24	11.21	16.97	62.5	1.82	2.42	7.67	10.0	−5.97

* 2 day period.

and occurred whenever one of the observers entered the room. It was not prevented by adequate oral and parenteral doses of atropine. Because of vomiting, sugar was stopped and the dog was given Ringer's solution fortified with potassium chloride and injectable vitamin B complex (solu-B, Upjohn) in an attempt to restore electrolyte balance and correct the obvious vitamin deficiencies. Dehydration became severe and the animal was almost moribund. Non-protein nitrogen on the last day of plasma injection was 63 mg. per cent. Generous amounts of hamburger steak followed by regular kennel ration restored the dog's weight and appearance of well-being, but proteinuria and occasional spontaneous hematuria continued for 3 months. The dog was killed with ether and autopsy revealed acute and chronic cystitis with a moderately severe chronic pyelonephritis. This condition was probably related to repeated catheterization in previous experiments and it helps explain the high figures for urinary nitrogen in periods 12 to 15, Table 8.

The *Summary Table* brings out the important points of the paper. It is to be emphasized that the first 5 tables (1 to 5) giving data procured by the use of three normal dogs, show a reasonably uniform response. The two bile fistula dogs, Tables 6 and 7, show differences but the fistula was probably not wholly responsible. The last experiment, Table 8, deals with an abnormal dog in which the cystitis and pyelonephritis was lighted up by the long period of protein fasting. A large negative nitrogen balance is to be noted.

It is apparent that larger amounts of plasma given parenterally will cause proteinuria to appear earlier and to a greater degree—compare two experiments on the same dog (43-141), in which the nitrogen of the injected plasma is increased by more than 50 per cent, the proteinuria appears on the 8th day (14th day with less plasma), and the percentage of injected plasma protein appearing in the urine measures 24 per cent (as compared with 16 per cent with less plasma).

SUMMARY TABLE

Table No.	Dog No.	Duration of plasma administration	N from plasma total	N from diet total	Urinary nitrogen			Urinary protein N as per cent of protein N injected	Time elapsed before proteinuria	Concentration of protein in plasma		
					Total	Protein	Balance			Proteinuria appeared	Maximal level	Average of all levels
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*days*	*gm. per cent*	*gm. per cent*	*gm. per cent*
1*	43-346	92	204	1.0	184	13.4	21	7	14	10.4	11.5	10.3
2*	43-346	76	178	3.0	154	14.0	27	8	26	10.3	11.5	10.0
3*	44-98	76	180	52.0	168	38.6	64	21	22	9.9	10.7	9.6
4	43-141	32	75	0.2	59	11.6	16	16	14	9.7	11.5	10.1
5	43-141	52	191	3.9	149	45.7	46	24	8	9.7	11.3	9.9
6	46-79	15	56	1.8	38	12.7	20	23	4	8.7	10.2	9.3
7	46-9	16	58	1.9	35	9.2	15	16	6	10.4	10.5	9.6
8	43-290	30	68	0	83	18.0	−15	26	8±	9.5	10.0	9.7

* See tables in preceding paper.
Tables 6 and 7—bile fistula dogs.
Table 8—cystitis and pyelonephritis.

There seems to be adequate evidence that there is a threshold for plasma proteins. The values for the initial proteinuria in terms of plasma protein concentration run from 9.7 to 10.4 gm. per cent in the normal dogs and the average figures in these same experiments are 9.6 to 10.3 gm. per cent—not a wide spread. The highest levels attained for plasma protein concentration are 11.5 gm. per cent. The overflow of protein is very considerable in some experiments—yet the proteinuria cleared in 1 to 4 days when plasma injections were discontinued, and the kidneys were demonstrably normal subsequently.

DISCUSSION

Our interest in the renal threshold for plasma proteins came about by accident. We attempted to repeat some earlier experiments (7) in which a pecu-

liar intoxication was observed in dogs receiving plasma by vein and sugar by mouth. As the repetitions did not bring on this intoxication, the experiments were lengthened and finally protein was found in the urine. The proteinuria increased as larger amounts of plasma were given parenterally, and when plasma injection was discontinued it promptly cleared. There was no evidence of renal damage following proteinuria at a high level maintained for 5 to 7 weeks. The first experiment is given in Table 4 and for contrast an animal with a diseased kidney (pyelonephritis—Table 8) was included in the series.

Renal thresholds for hemoglobin in the dog have interested workers in this laboratory for years. Thresholds for dog hemoglobin, for dog muscle hemoglobin, for goose and sheep hemoglobin have been established (12). The threshold for muscle hemoglobin was about 5 per cent that of blood hemoglobin. The renal threshold for blood hemoglobin can be lowered by repeated injection of hemoglobin day by day, which results in accumulation of it in the epithelium of the convoluted renal tubules (11). Further study of hemoglobin renal clearance is reported by Monke and Yuile (13, 21, 22).

It is obvious that the small protein molecules pass the renal threshold in greater amounts—albumin 3 or 4 times that of the globulins in these experiments. *Fibrinogen* stands out as distinct from the other globulins by present methods of analysis. Fibrinogen may in fact pass the glomerular tuft, be precipitated as fibrin in the tubules, to be absorbed or perhaps digested by the tubular epithelium (not unlike some hemoglobin in hemoglobinuria). The amounts of fibrinogen concerned would be very small and could readily escape detection.

Some may believe that the renal threshold is wholly dependent upon the level of plasma proteins in the blood and this may be a fact. However, we must consider the possibility of some absorption of proteins by the convuluted tubular epithelium, which might for a time delay the appearance of proteinuria.

SUMMARY

Proteinuria in normal dogs can be produced at will by parenteral injections of dog plasma.

As the plasma injections are continued the plasma protein concentration rises and at some point protein begins to appear in the urine. The level of plasma protein concentration at which proteinuria appears in normal dogs ranges from 9.6 to 10.4 gm. per cent. This may be termed the renal threshold for proteinuria. Repeat experiments in the same dog show threshold levels to be practically identical.

An interval of days (4 to 26 days) has been noted between the start of plasma protein injections and the appearance of the proteinuria. Larger doses of plasma shorten this interval and the critical plasma protein level is attained sooner.

Considerable amounts of protein may appear in the urine—298 gm. protein during a 52 day period in one instance studied—yet the urine clears in 1 to 4 days after cessation of protein injections. Autopsy shows undamaged kidneys.

Maximal levels of plasma protein concentration range from 10.0 to 11.5 gm. per cent. The highest levels are usually associated with maximal output of protein in the urine.

It seems clear that plasma proteins readily pass cell barriers (or membranes) within the body, including the endothelium and epithelium of the renal glomerulus.

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TUBERCULOSIS OF RABBITS INDUCED BY DROPLET NUCLEI INFECTION

I. Initial Response to Infection*

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Plates 9 to 14

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The significance of droplet nuclei infection in the spread of bacterial and virus diseases has been established by experimental and epidemiological studies (1–3). Experiments also have demonstrated that transport of droplet nuclei infection into the alveolar tissues of the lungs by the air currents of normal respiration depends upon the settling velocity of droplet nuclei (4). The experiments by which the relation of settling velocity of droplet nuclei to their transport by the air currents of normal respiration was determined used virulent tubercle bacilli as indicator organisms and rabbits as test animals. These animals inhaled the organisms as separated cells in droplet nuclei. Thus the tubercles which developed in their lungs were known to be induced by organisms derived from single cells, all of which were implanted upon alveolar tissue within relatively brief intervals.

During a period of about 6 weeks following infection these tubercles developed at a remarkably uniform rate which was not appreciably influenced either by the number of lesions or by their position in the lungs (4). These observations are consistent with the results of similar experiments which involved rabbits of families of high and low levels of resistance to tuberculosis (3). Thus it seems that the initial response of normal rabbits to virulent bovine tubercle bacilli, inhaled as separated cells in droplet nuclei, may be said to be homogeneous. The homogeneous phase of inhaled tuberculosis, as observed in these experiments, contrasts sharply with later stages of the infection which are well known to be strikingly heterogeneous, especially when rabbits of various strains are subjected to infection. This observation suggests that rabbits do not differ in their inherent resistance to bovine tubercle bacilli. Instead, they differ in their capacity to acquire resistance. Additional evidence which supports this hypothesis is provided by a study of the sequence of histological changes during the homogeneous phase of inhaled tuberculosis and of its transition into the

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heterogeneous phases of the reaction. These features of air-borne tuberculosis will be described in this paper.

Material and Methods

Animals were infected by organisms of the Ravenal strain of *Mycobacterium tuberculosis (bovis)* which had been grown in a liquid medium in rotating flasks containing glass beads. This method has been found to stimulate rapid growth of the organisms, to a large extent in the form of separated cells (5).

Infections were induced by newly developed methods and apparatus. This apparatus consisted of an aerosol flask, an inhalation chamber, and an incinerating chimney connected in series. Animals exposed in this apparatus inhaled virulent organisms, air-borne as separated cells in fine droplet nuclei, under standardized conditions (6). The procedure for a typical experiment was as follows:—

Aqueous suspensions of separated organisms were prepared by passing a liquid culture through No. 4 Whatman filter paper. Density of organisms in this filtrate was determined by the Breed method, which had been found to give results consistent with colony counts of suspensions planted on the Crumb medium (7). Then the filtrate was diluted to a level which would provide the desired concentration of bacilli in the aerosol, and the dilute suspension introduced into the aerosol flask.

The aerosol flask was made from a Florence flask by blowing an auxiliary neck tangential to its equator. This flask was attached in the horizontal position by its main neck to the intake portal of the system, with the auxiliary neck rotated above the level of the bacterial suspension. A Venturi nozzle and throat, carefully designed for efficient atomization, was fitted into the auxiliary neck of the flask. The base of this nozzle extended to the exterior of the flask through a rubber stopper to connect with a tank of compressed air. Adjustments being completed, the flask was rotated on its horozintal axis to bring the fluid into the auxiliary neck about the base of the Venturi throat to allow the bacterial suspension to enter. Opening the valve to the air line allowed a high velocity jet of air to draw fluid from the Venturi throat and whirl it into a cloud of droplets about the equator of the flask. Larger droplets impinged upon the wall of the flask and returned to the pool of liquid. Only the smallest ones evaporated rapidly enough to produce droplet nuclei which were carried by the air stream from the core of the cloud into the system through the horizontal neck of the flask. Occasional droplet nuclei contained tubercle bacilli.

The aerosol passed from the flask into the inhalation chamber through a large duct which entered the bottom of this chamber beneath a false floor. This floor was perforated at its periphery. The inhalation chamber, a hexagonal metal box, accommodated a modified Murphy respirator on each of its sides. Rabbits were placed in the respirators with the rubber collars about their necks. When the respirators were bolted in place, these collars served as gaskets to seal the apertures. This arrangement admitted only the heads and necks of the animals into the inhalation chamber in positions above the perforations in the false floor, while the air flowed from beneath upward and, after passing the noses of the rabbits, was drawn downward through a central outlet to the exhaust duct. The exhaust duct connected with the incinerating chimney, which served to sterilize the aerosol and to maintain negative pressure within the system.

The concentration of organisms in the aerosol and the length of exposure determined the intensity of infection. When an exposure had been completed and atomization was stopped, the system was cleared by ventilation and ultraviolet irradiation, after which the animals were removed and returned to their cages.

Fifty-six albino rabbits, all purchased from one source, were used in these experiments. These animals weighed about 2 kilos each when they were exposed to infection. They were

kept in individual cages and fed a commercial mixture supplemented by carrots. All of them harbored species of *Eimeria*, but accidental bacterial disease was not found.

Animals which were killed for study were bled from the carotid artery. When respiration had stopped, the trachea, lungs, and heart were removed with intercommunicating channels intact and the lungs were partially deflated by immersion in formalin diluted to 6 per cent in 0.85 per cent aqueous sodium chloride solution. Following partial deflation the lungs were moderately distended by introducing fixative into the trachea through a pipette and bulb. Repeated distention and immersion into the fixative removed the greater part of the alveolar air, after which the trachea was clamped and the organs fixed for several days. Lungs of rabbits that died of tuberculosis were also fixed in this way, but rarely could they be so completely filled by the fixative. As judged by histological appearances, however, fixation was equally satisfactory.

Sections were cut at 5 microns and stained by carbol fuchsin and Mayer's hemalum. The number of blocks of tissue taken from any set of lungs for section varied with the character of the disease. In the study of the early stages of the infection, for example, blocks of tissue were taken from each lobe of the lungs and 10 to 20 serial sections cut from each block. Each of these series was estimated to contain 5 to 10 c.mm. of tissue.

EXPERIMENTS

1. The Initial Reaction of the Lung to Air-Borne Tubercle Bacilli

This phase of the study utilized 16 rabbits, each of which was estimated to have inhaled upwards of 20,000 organisms. Six of the animals were infected as one exposure group and killed in pairs, 1, 2, and 3 days after infection. Other animals were infected in pairs and killed in pairs 2, 5, 6, 9, and 12 days after infection.

Under the conditions of the experiment bovine tubercle bacilli, deposited in the lungs of previously uninfected rabbits, as individual cells, did not excite inflammatory response for about 12 days. During this interval the organisms became increasingly abundant within the alveolar macrophages, as seen in sections, without inducing visible changes in these cells or in the surrounding tissues.

Tubercle bacilli were found in each of the series of sections cut from the lungs of these rabbits. But, in the sections from animals killed within 7 days after infection, prolonged search was required. The lungs of these animals, when filled but not forcibly distended by fluid, displaced about 50,000 c. mm. Since about one-half of this volume was occupied by bronchi, bronchioles, and blood vessels upon which the tubercle bacilli apparently did not gain a foothold, the inhaled organisms were deposited in about 20,000 c. mm. of susceptible lung tissue.

1st Week.—During the 1st week after infection the tubercle bacilli invariably were found in isolated alveolar macrophages which were more or less loosely attached to the walls of otherwise normal alveoli, and recognizable by carbon particles in their cytoplasm. As a rule that part of the cytoplasm of the macrophage included in a section contained a single, solidly stained bacillus. Oc-

casionally, however, 2 or 3 organisms were found in a section of a cell. The average frequency of infected macrophages was upwards of one in 200 sq. mm. of tissue section. Since sections approximated 5 microns in thickness, this number corresponded to the number of bacilli estimated to have been inhaled; *i.e.*, one organism per cubic millimeter. The frequency of infected macrophages did not increase appreciably during this 1st week of infection.

2nd Week.—Between 6 and 9 days after infection, however, the numbers of infected macrophages seemed to have increased by about tenfold. In the lungs of animals killed 9 days after infection a majority of the infected cells still were isolated macrophages (Fig. 1). Less frequently the infected cells were found in small groups (Fig. 2). Alveoli containing the infected cells were unchanged otherwise.

Between 9 and 12 days after infection the collections of parasitized macrophages, as seen in serial sections of the lungs of 4 rabbits killed at these intervals, seemed to have increased rapidly in size. As judged by this material, the rate of increase in the size of these foci of infection was more pronounced during this interval than from 6 to 9 days, and the numbers of bacilli within these foci were increased correspondingly (Figs. 3 and 4). This apparently accelerated development of the infection was in keeping with progressive growth of the organisms, if this followed approximately the expected normal rate.

After 12 days of development a majority of infected foci occupied only one or two alveoli, but occasional ones had spread into several adjoining air spaces. Usually the alveoli were compactly filled by alveolar macrophages, which fused into dense masses and sometimes formed giant cells which resembled cross-sections of poorly defined tubules (Figs. 5 and 6).

At this time leukocytes and small monocytes had begun to accumulate about the developing tubercles. In some lesions numerous leukocytes clustered about the infected macrophages and penetrated into the masses of cells. The small monocytes were most conspicuous in the alveolar walls about the infected cells where they had become sufficiently numerous to thicken the tissue.

In addition to the reaction which has been described, scattered acute focal inflammatory lesions developed in the lungs of more than half of the animals in this series. These foci were well developed in 24 hours in one rabbit and seemed to be disappearing after 3 to 6 days, as judged by appearances in sections of lungs examined at these intervals (Figs. 7 and 8). In some animals they were found in all parts of the lungs; in others only one or two series of sections contained them. Their centers were composed of alveolar macrophages, with scattered leukocytes among these cells and about their outer borders. More abundant than the leukocytes were small, dark staining cells which resembled lymphocytes. These cells were most numerous about the periphery of the lesions, apparently having moved into the foci from blood vessels and capillaries.

An explanation for these lesions is not apparent. The rabbits inhaled from 10 to 100 uninfected droplet nuclei for every one which contained a bacillus.

If these lesions were reactions to the uninfected droplet nuclei they should have been much more abundant and more uniformly distributed. But they were sparsely developed and irregularly distributed. Hence they are considered to be non-specific reactions. They are described because it was inevitable that occasional ones would be infected, in view of the numbers of organisms inhaled by these animals. Apparently, even when infection occurred, these non-specific foci underwent some degree of regression, but the alveolar macrophages and the small, dark cells persisted in small numbers as the bacilli increased. The progress of these lesions seemed to correspond to that of ones which apparently developed from simple infection of isolated alveolar macrophages. This opinion is based upon study of infected non-specific foci found in the lungs of one rabbit killed 72 hours after infection, and of another killed 9 days after infection (Fig. 9).

2. Development and Progress of Initial Tubercles

Beyond the 2nd week of its development the response of the lungs to initial infection by separated bovine tubercle bacilli has been studied in 40 rabbits. Fourteen of these died or were killed between 16 and 28 days, 14 between 32 and 43 days, 6 between 56 and 72 days, and 6 after 100 or more days of infection. The number of organisms inhaled by these animals during the single exposure to which they were subjected varied, by the plan of the experiment, from less than 10 to more than 20,000.

3rd Week.—The tubercles became visible by the end of the 3rd week as translucent, pale foci, 1 to 2 mm. in diameter. The histological preparations suggested the following pattern of development during this period.

Growth of the bacilli in the alveolar macrophages continued unchecked and these masses of infected cells spread to adjacent alveoli through the alveolar ducts. With this growth an inflammatory reaction developed rapidly about the foci of bacterial growth. Within 18 to 20 days alveolar macrophages had disappeared as intact cells to leave the bacilli concentrated among their fragments while leukocytes and monocytes or histiocytes continued to move into the mass and distintegrated (Fig. 10).

The spaces which contained these centers of bacterial growth often were lined by flattened basophilic cells, apparently monocytes. Alveolar walls immediately about the centers became increasingly thickened and alveolar spaces filled by monocytes among which were smaller number of leukocytes and lymphocytes. Occasional monocytes near the inner border of the mass of inflammatory cells contained bacilli.

By the end of the 3rd week of growth the tubercles consisted of relatively wide inflammatory zones centering about the spaces in which the bacilli were concentrated. Toward the periphery the alveoli tended to be free of cells although alveolar walls were infiltrated well beyond this zone.

4th Week.—In the lungs of rabbits killed 28 days after infection tubercles

appeared as moist, translucent nodules, 4 or 5 mm. in diameter, outlined by narrow borders of injected capillaries. In sections the greater part of the nodules was composed of the zone of inflammatory cells. The necrotic centers seemed to have expanded less rapidly and, in expanding, seemed to follow alveolar ducts, so that their outlines became irregular. This expansion of the necrotic centers into alveolar ducts seemed to account for the contact between them and the lumina of the terminal bronchioles (Fig. 11). However, evidence of bronchial spread of the infection was not found until much later (Fig. 12).

Late in the 4th week of infection spread of the organisms from the initial lesions along perivascular and peribronchial lymph channels was found and, within this period, epitheloid tubercles developed in the tracheobronchial lymph nodes of some animals.

5th and 6th Weeks.—Within the 5th week after infection all tubercles underwent more or less extensive central caseation which separated the masses of bacilli and necrotic cells in the core of the lesions from the cells of the inflammatory zone. During this time the rate of peripheral expansion of the tubercles decreased; apparently with caseation fewer macrophages and leukocytes migrated into the mass, and the width of the caseous zone came to exceed that of the inflammatory zone. During the 5th week, also, differences in the rate of progress of the tubercles from animal to animal became evident, although in the lungs of any one animal all lesions were remarkably uniform in size.

On the whole, smaller tubercles seemed to be most completely caseous and, in these, the bacilli were more distinctly concentrated in the central zone. Often organisms could be found only in these central zones, enclosed in spaces which corresponded to alveolar ducts and were outlined by poorly stained reticulum fibers.

By the end of the 6th week of infection differences in the rate of progress of the disease from animal to animal were well developed. The reaction to infection had become heterogeneous. Thus in the lungs of some of the rabbits which were killed 43 days after infection, tubercles were scarcely larger than at 5 weeks. They were well circumscribed, with extensive central zones of caseation and relatively narrow borders of inflammation. Evidence of spread of the organisms by way of the bronchi was not found, nor had tubercles developed in other organs. Other animals at 6 weeks had well developed secondary tubercles both of the lungs and kidneys. In the lungs, caseous material containing large numbers of bacilli apparently had drained into the connecting bronchioles and had been deposited in adjacent parts of the lung. This resulted in the initial lesions being surrounded by clusters of satellite tubercles. Or the infection had spread by the blood stream and many small tubercles had formed where bacilli had chanced to lodge in the lungs and kidneys (Figs. 13 to 16). The size of the secondary tubercles which were found after 6 weeks

of infection suggested more than 2 weeks' growth but, without knowing the numbers of organisms by which such lesions were initiated, there was no basis for an estimate of age.

These differences in the rate of progress of the infection during the 5th and 6th weeks sometimes could be related to the intensity of initial infection. Three of 6 rabbits which had inhaled approximately 150 bacilli each, exhibited macroscopic secondary tubercles of the lungs and kidneys when they were killed 43 days after infection. In 6 other rabbits, each of which had inhaled about 10 bacilli, the infection was limited to the initial tubercles except for spread to the tracheobronchial lymph nodes.

Later Phases.—Progress of individual tubercles in the lungs of any rabbit varied after 6 or 7 weeks. Some tubercles in the 5 rabbits killed 7 to 10 weeks after infection had not expanded beyond diameters of 5 or 6 mm. Others had enlarged to about 10 mm. in 10 weeks (Figs. 17 and 18). The expanding ones, more often than not, were located beneath the pleura and tended to enlarge along this surface rather than in other directions. The pleura was considerably thickened by fibrosis but other surfaces of the lesions were bounded by dense zones of epitheloid cells among which were smaller numbers of lymphocytes, monocytes, and leukocytes.

The smaller tubercles were sharply circumscribed in the tissue while the larger ones were associated with numbers of satellite lesions. Unless there was obvious evidence of drainage of the caseous centers into bronchioles the numbers of demonstrable bacilli in the initial tubercles at 10 weeks were less than 10 per cent of the numbers present at 5 or 6 weeks. Likewise, the numbers of infected monocytes among the cells of the inflammatory zone seemed to have been reduced in these relatively inactive tubercles. These estimates were based upon study of serial sections taken through the centers of a number of representative lesions.

When, however, the tubercles were draining into bronchi, the numbers of bacilli within them were enormously increased. At the same time the zone of inflammation about the periphery of the initial lesions decreased and they became thin walled cavities into which leukocytes and monocytes migrated, to disintegrate among the masses of bacilli. Fibrosis about such cavities was very slight.

The 6 rabbits examined more than 100 days after infection all carried more than 30 initial tubercles, and all had developed a number of cavities (Figs. 19 and 20). Usually these were located in the dorsal parts of the lungs and the infection apparently spread, mainly by gravity, to the dependent parts of these organs. However, some of the cavities, also, were located on the ventral surfaces.

Among these 6 animals there were striking differences in the rate of spread

of the infection. However, the rate of progress of the infection did not seem to be related to the major pathway of spread, which, in these animals, was the bronchi rather than the blood stream.

DISCUSSION

Under the conditions of these experiments the reaction between the lungs of normal rabbits and virulent bovine tubercle bacilli, inhaled as separated cells in droplet nuclei, was completely homogeneous for about 4 weeks. The separated bacilli were ingested by alveolar macrophages and seemed to grow progressively in these cells without causing obvious damage to them for about 2 weeks. Apparently infected cells did not wander from alveolus to alveolus to an appreciable degree. Instead, other alveolar macrophages, recognizable by carbon particles in their cytoplasm, moved into the alveoli in which the bacilli had been deposited. How these cells came to contain organisms, unless the macrophages which were infected originally, disintegrated, cannot be imagined. Yet no cell which seemed to be degenerating was found in the infected alveoli before 12 days had passed.

Tubercle formation may be said to have started about the end of the 2nd week of the infection. At this time the alveolar macrophages had fused into imperfect giant cells or had become vacuolated. Progressive development of the inflammatory reaction and growth of the bacilli apparently continued unchecked until the tubercles underwent caseation during the 5th week of infection. Thereafter the reaction to the infection became heterogeneous. No evidence of delayed tubercle formation was encountered; this has been postulated to occur when air-borne infection was induced by another technique (8).

Differences in the rate of progress of the infection after 5 or 6 weeks did not seem to be related to differences in the character of the inflammatory response. Instead, heterogeneity of the pattern of disease could be related to the intensity of the inflammation which, in turn, seemed to correspond to the differences in the growth rate of the bacilli after the 4th week, these differences, of course, being estimated from the number of organisms found in the lesions. This, it must be admitted, was a crude measure at best. Yet careful study of serial sections of representative tubercles revealed differences in the numbers of demonstrable bacilli of such magnitude that more exact methods hardly seemed necessary. It appears worth considering that heterogeneity of progressive tuberculosis may be related to differences in the capacity of animals to change the composition of their tissues or blood in such a way that growth of the bacilli may be more or less inhibited.

The original purpose of this study was to evaluate tubercle bacilli as indicator organisms in investigations of the dynamics of droplet nuclei infection (4). When it became evident that, under the conditions of these experiments, the rate of development of initial tubercles did not vary with the animal until after 5 or 6 weeks of growth, a more complete study of the early phases of air-borne tuberculosis seemed advisable.

The homogeneous phase of tuberculosis, as disclosed by this study, seems to have been overlooked in most investigations of the immediate response to infection (9–12). Possible reasons for this are: (1) methods used to induce infections may have implanted more than single organisms in the average focus, (2) the rate of tubercle development is not the same in all tissues, and (3) the rate of tubercle development may differ with the species. Furthermore, animals may react to the medium in which the bacilli are suspended, or non-certain specific reactions may be confused with the response to the infection. Therefore it seems unwise to attempt to compare the results of different experiments, unless infection techniques be equally precise, and the response to infection studied in the same tissue. However, by the use of methods which approximated those of the present experiments, the early response of guinea pigs to inhaled tubercle bacilli has been found to correspond to that of rabbits as here reported (13).

SUMMARY AND CONCLUSIONS

Rabbits were caused to inhale known numbers of virulent bovine tubercle bacilli as separated cells in droplet nuclei.

For approximately 5 weeks after infection the growth of the bacilli and the response of rabbits to their growth was homogeneous; *i.e.*, all reacted in the same way and to the same degree.

After 6 weeks individual differences in the rate of progress of the initial tubercles and of the infection as a whole became evident. These variations in the response seemed to be influenced by the number of initial tubercles and by the number of bacilli found in the lesions.

It is concluded that, as evidenced by the homogeneous phase of infection, rabbits do not differ in their resistance to initial growth of bovine tubercle bacilli. However, the later, heterogeneous pattern of response suggests that these animals vary widely in their capacity to acquire resistance.

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EXPLANATION OF PLATES

Photography by Mr. Basil Varian, Department of Anatomy, University of Pennsylvania.

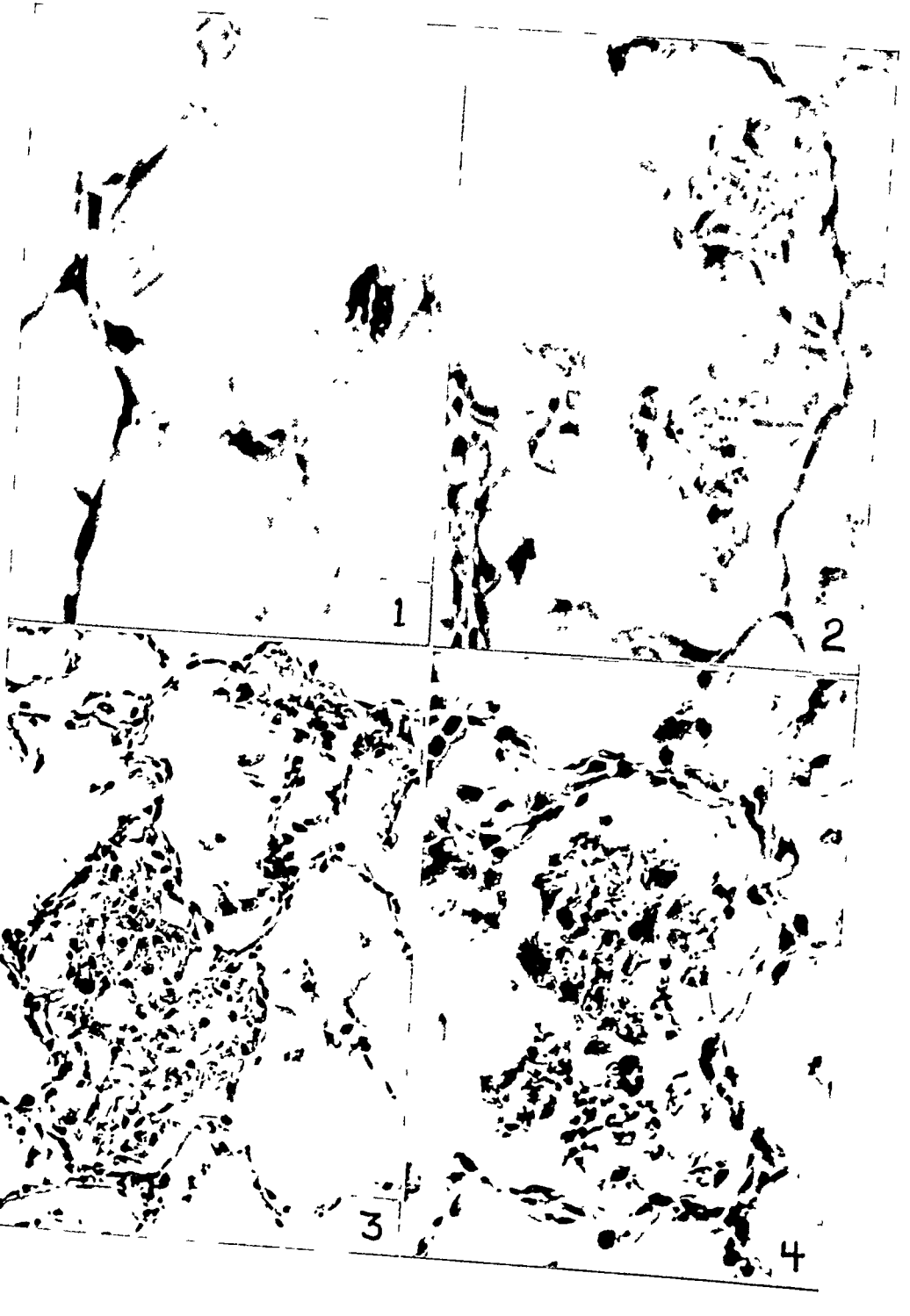
PLATE 9

FIG. 1. Rabbit 14-8. Tubercle bacilli in the cytoplasm of an alveolar macrophage 9 days after inhaled infection. × 1100.

FIG. 2. Rabbit 14-7. A group of infected alveolar macrophages 9 days after inhaled infection. The bacilli are distributed as solid black rods among these cells which also contain carbon particles. The small cells of this alveolus appear to be monocytes. Note that the alveolar walls about the infected cells in Figs. 1 and 2 are unchanged. × 700.

FIG. 3. Rabbit 14-4. A focus of infection after 12 days' development. The alveolus is filled chiefly by alveolar macrophages, a majority of which contain bacilli. Monocytes are present in small numbers among and about these cells and have accumulated in the walls of the adjacent alveoli. × 400.

FIG. 4. Rabbit 14-4. An infected focus after 12 days' development. This illustrates the accumulation of leukocytes and monocytes about the cluster of infected macrophages. × 600.

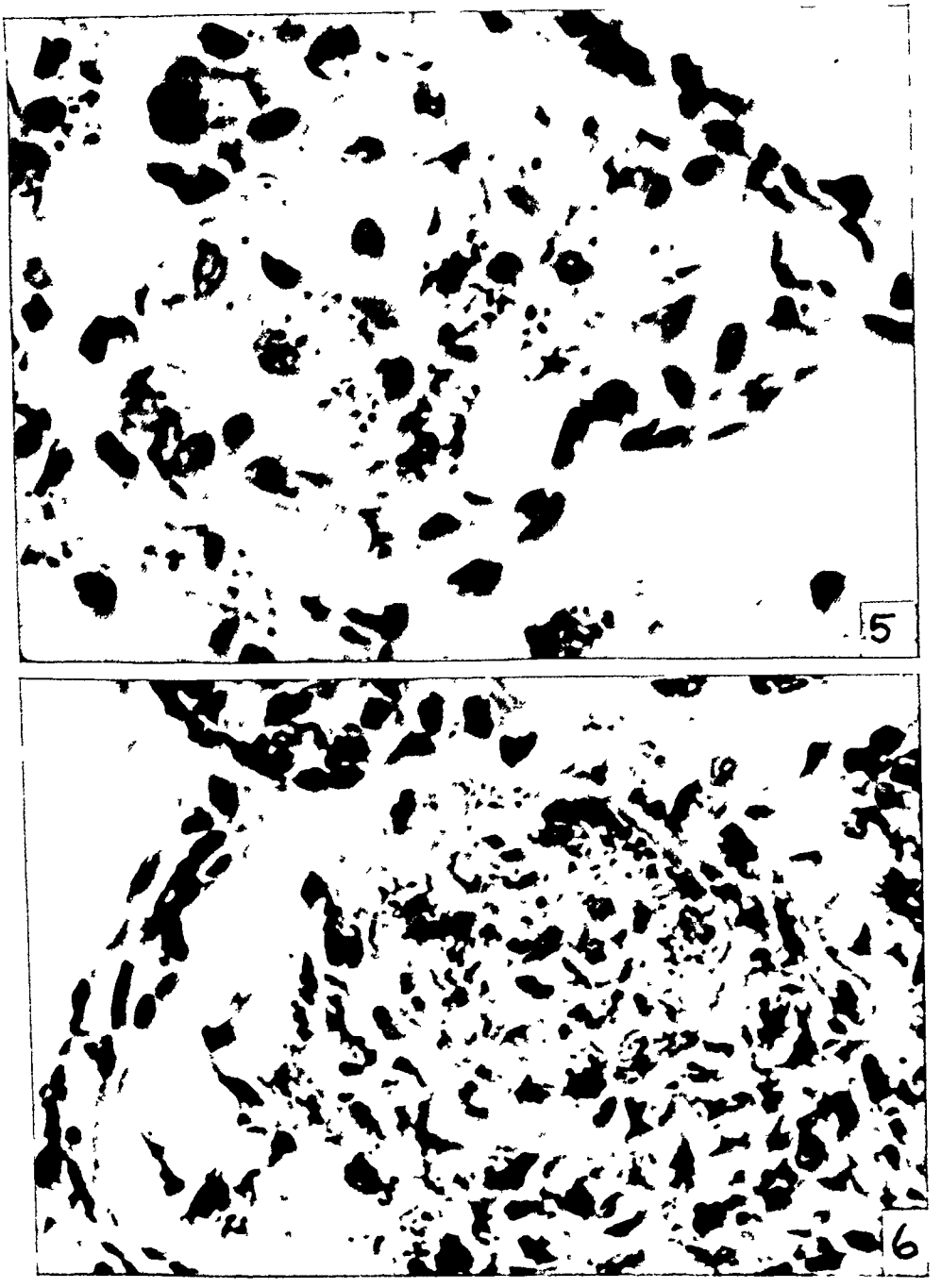


(Ratcliffe and Wells Tuberculosis of rabbits. I)

PLATE 10

FIG. 5. A part of the focus of infection shown in Fig. 3, at a higher magnification. Bacilli and carbon granules are seen within the cytoplasm of the fused mass of alveolar macrophages. The upper and lower right edges of this mass of infected cells are bordered by the thickened wall of the alveolus. × 1100.

FIG. 6. Another infected focus from rabbit 14-4, 12 days after infection, illustrating the fusion of infected alveolar macrophages to form giant cells. Carbon granules and bacilli are scattered through this fused mass of cells. Monocytes and leukocytes surround the mass. × 1100.



(Ratcliffe and Wells Tuberculosis of rabbits I)

PLATE 11

FIG. 7. Rabbit 10-1. A focus of non-specific reaction, 24 hours after the animal began inhaling the aerosol suspension of bacilli. Note that this focus is more extensive than a developing tubercle 11 days older. Serial sections of this focus did not reveal bacilli. × 400.

FIG. 8. Rabbit 10-4. A focus of non-specific reaction, 72 hours after the animal began inhaling the aerosol suspension of bacilli. At this time these foci were less compact and appeared to be breaking up. This focus did not contain bacilli. × 500.

FIG. 9. Rabbit 14-8. Tubercle bacilli in the cytoplasm of alveolar macrophages 9 days after infection. This lesion, and several others like it in the lungs of this animal, were attributed to chance infection of a focus of non-specific reaction. Compare the thickened alveolar walls about this group of cells with those shown about the average infected focus after 9 days' development (Figs. 1 and 2). × 500.

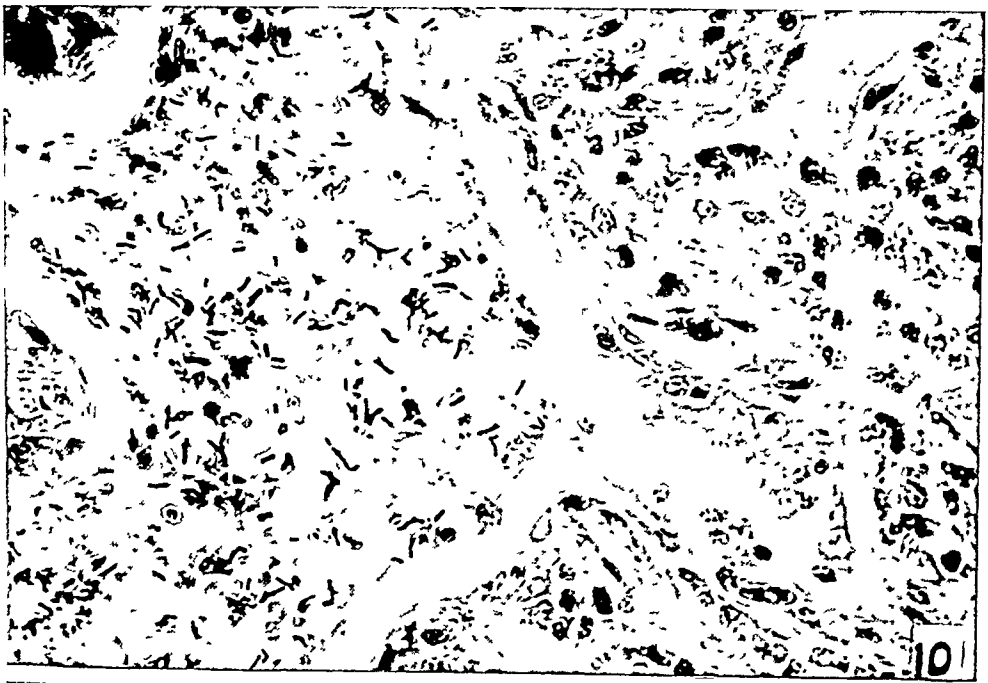
(Ratcliffe and Wells Tuberculosis of rabbits. I)

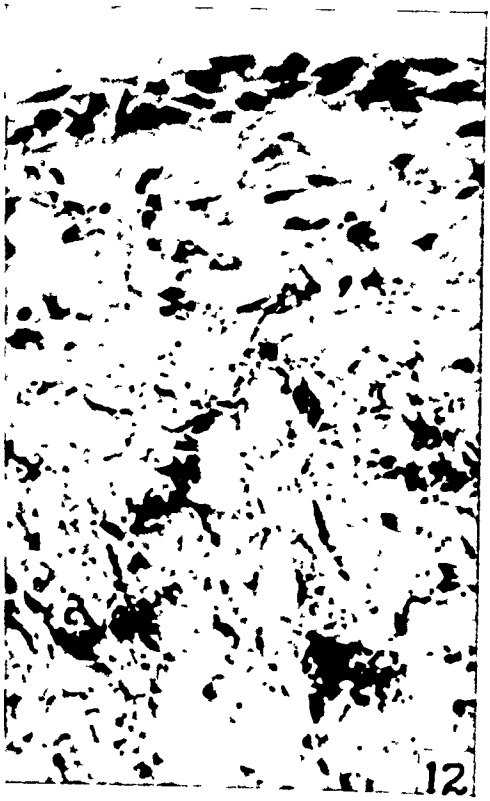
PLATE 12

FIG. 10. Rabbit 5-4. Killed 20 days after inhaling 50 to 75 bacilli. This field is representative of the centers of initial tubercles during the latter part of the 3rd week and early part of the 4th week of development. Bacilli were found mainly among the cells which filled alveolar spaces and alveolar ducts. × 600.

FIG. 11. Rabbit 10-8. Killed 28 days after inhaling about 150 bacilli. This field illustrates the communication between the necrotic center of the tubercle, which contained abundant bacilli, and a patent bronchiole. × 350.

FIG. 12. Also from rabbit 10-8. This illustrates the lack of reaction in the wall of a large bronchiole as the expanding tubercle encroached upon it. Bacilli make up the black masses scattered through the lower two-thirds of the field. × 660.





(Ratcliffe and Wells. Tuberculosis of rabbits. I)

PLATE 13

Photographs of lungs are natural size.

FIG. 13. Rabbit 9-1. Killed 39 days after inhaling 175 to 200 bacilli.

FIG. 14. Rabbit 11-6, killed 43 days after inhaling 10 to 20 bacilli.

FIGS. 15 and 16. Rabbits 10-9 and 11-2, killed 43 days after inhaling 150 to 175 bacilli. Fig. 14 illustrates the average character of initial tubercles at 6 weeks, when small numbers of bacilli were inhaled.

Figs. 13 and 16 illustrate progression of the infection by the air passages, and Fig. 15, progression by both air passages and blood stream at 6 weeks when initial infection was relatively intense. The irregular borders of the initial tubercles of Figs. 13, 15, and 16 could be traced to local spread of the infection through alveolar ducts and bronchioles.

(Ratcliffe and Wells Tuberculosis of rabbits I)

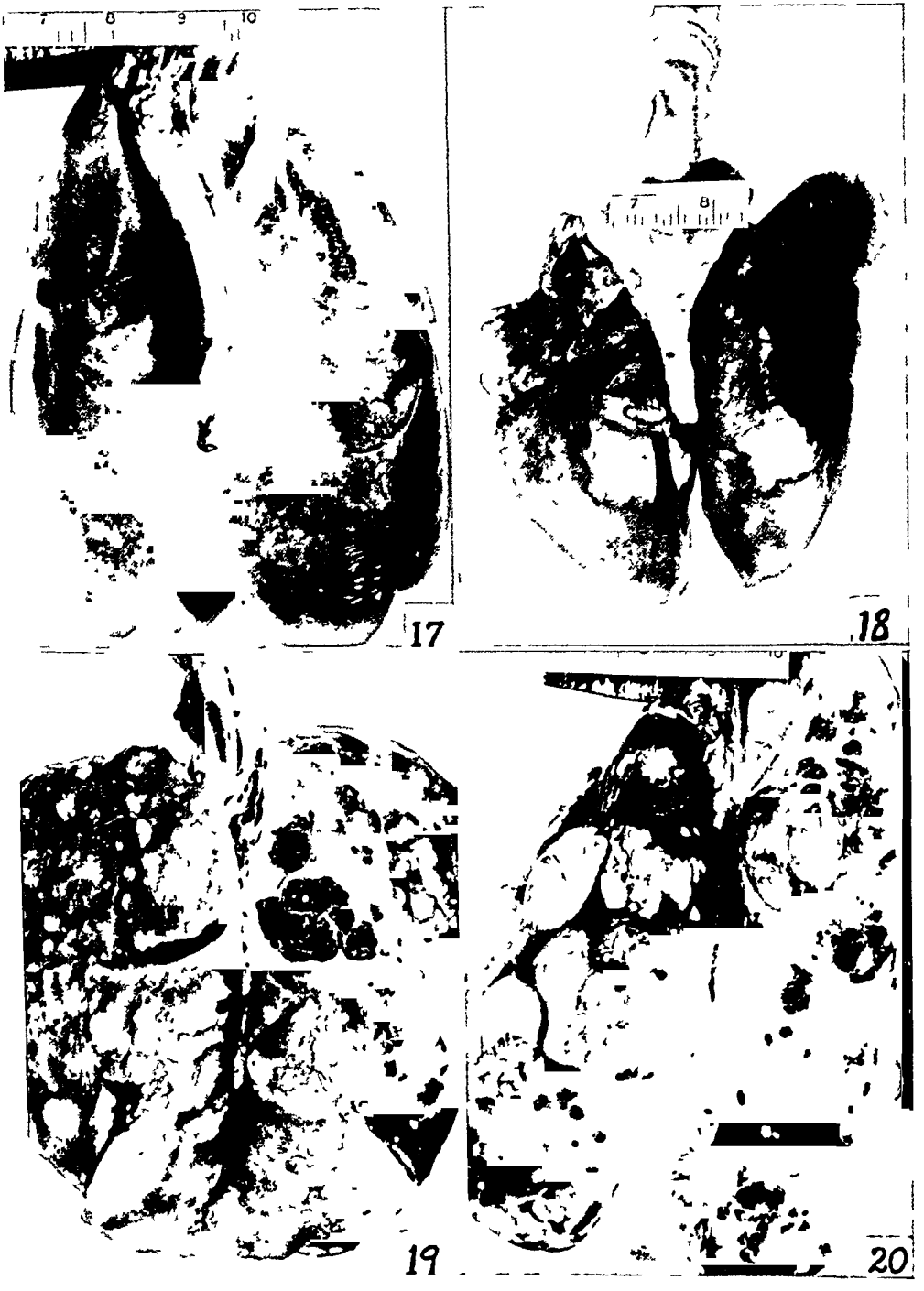
Plate 14

Fig. 17. Rabbit 15-8, killed 56 days after infection by 5 air-borne bacilli. The tubercles in this animal are representative of the relatively slow progress of the infection at this time interval, when small numbers of bacilli were inhaled. Spread of the infection along the pleural lymphatics from the initial tubercles is clearly illustrated.

Fig. 18. Rabbit 7-4, killed 71 days after infection by 5 air-borne bacilli. The initial tubercles are correspondingly larger than those of Fig. 17, and bronchial spread of the infection is shown by satellite tubercles near the initial lesions and by small tubercles scattered through the lungs. Other organs were not involved.

Fig. 19. Rabbit 9-5, killed 132 days after infection by 175 to 200 air-borne bacilli. This animal was an exposure-mate of rabbit 9-1, Fig. 13. Some initial tubercles had advanced to form cavities; others were no larger than at 6 weeks; and still other initial tubercles seemed to have regressed. In spite of relatively intense initial infection this animal apparently developed a high grade of resistance to progression of the infection.

Fig. 20. Rabbit 12-3, died of perforated tuberculous ulcer of the gut 121 days after inhaling about 30 bacilli. Cavities account for about one-half of the initial tubercles. The remainder apparently regressed.



(Ratcliffe and Wells Tuberculosis of rabbits I)

TUBERCULOSIS OF RABBITS INDUCED BY DROPLET NUCLEI INFECTION

II. Response to Reinfection*

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Plates 15 to 17

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When normal rabbits were caused to inhale an aerosol suspension of virulent bovine tubercle bacilli, the rate and pattern of tubercle formation were highly uniform for about 5 weeks. Thereafter, the progress of the disease was not uniform; it varied with the animal.

Transition of this relationship from the homogeneous phase to one in which the progress of the disease varied with the animal has been attributed to the development of resistance (1, 2). As judged by differences in the rate of development of initial tubercles after the 5th week of growth, it appeared as if resistance developed slowly and the rate of its development varied widely. After the 5th week of infection the rate of growth of the initial tubercles and extension of the infection to other foci was proportional to the number of bacilli contained in the lesions. Thus the only demonstrable effect of developing resistance seemed to be upon the apparent growth rate of the bacilli (2).

Experiments on inhaled reinfection have provided another method by which the effects of developing resistance upon the rate of growth of the bacilli may be observed. These experiments have shown that demonstrable levels of resistance develop more rapidly and increase at a more uniform rate than the study of initial infection had indicated.

Material and Methods

The 36 albino rabbits which were used in these experiments were purchased from the dealer who had supplied animals for other studies of this series. These animals weighed about 2 kilos each when they were first subjected to infection. All of them harbored species of *Eimeria* but accidental bacterial disease was not encountered. Details of feeding and care of these animals, as well as the methods by which infections were induced and tissues prepared for study, have been described fully in earlier publications (1–5).

EXPERIMENTS

Preliminary experiments demonstrated that rabbits exposed to massive reinfection 6 or more weeks after initial infection did not respond as do normal animals. Accordingly the response to reinfection was further studied by rein-

* This work has been supported by a grant from the Commonwealth Fund to the University of Pennsylvania for studies on the prevention and control of air-borne infection.

fecting rabbits at intervals from 2 to 7 weeks after initial infection. To this end, 18 normal rabbits, in 3 exposure groups of 6 animals each, were allowed to inhale about 30 bacilli per animal and divided into 6 groups for reinfection. Each reinfection group contained one rabbit from each of the 3 initial infection groups and one normal control.

The first of these groups was reinfected 2 weeks after initial infection. Each week thereafter another group was reinfected until all had had a second exposure, during which every animal inhaled upwards of 20,000 bacilli. This large dose invariably killed normal animals within 4 weeks. Following the second exposure to infection each group of rabbits was observed until the control animal had died. If, by this time, no other member of the group had died of tuberculosis, one of the reinfected rabbits was killed for examination. With 2 exceptions (see Table I), other reinfected rabbits were held until dead.

1. Time Response to Reinfection

The results of this experiment have been summarized in Table I in which the animals are arranged by reinfection groups, whereas they had been numbered in regular sequence for the initial infection. This table shows the intervals in days between infection and reinfection, and reinfection and death. Under the heading "Character of the disease" a brief description is given of the macroscopic features of the disease in each animal.

All control rabbits of this series died of tuberculosis within 4 weeks after infection. At death their lungs were fully expanded, deeply congested, and friable. Closely placed tubercles, many of which had fused, were distributed throughout these organs, and usually occupied more than half of the tissue (Fig. 1). With reference to size, cellular components, and numbers of bacilli, these lesions corresponded in all essential features to other initial tubercles of the same ages (Figs. 3 and 5). Their development apparently had not been influenced by the number of organisms inhaled.

The appearance of the lungs of rabbits of the first, second, and third reinfection groups which died or were killed within 32 days after reinfection, was much the same as that of the lungs of the control rabbits. The closely placed reinfection tubercles seemed to have developed at about the same rate as did initial tubercles. The larger initial tubercles, distributed at random through the lungs, also seemed to have progressed at about the rate that would have been expected, if these rabbits had not been reinfected (Fig. 2). However, histological changes in the lungs of these animals suggested that initial tubercles were affected by reinfection, and that reinfection tubercles were influenced by initial infection.

Initial tubercles of these animals were outlined by poorly defined, narrow borders of monocytes and leukocytes which surrounded relatively large irregular caseous centers. In contrast, initial tubercles, of the ages at which these lesions of reinfected animals were examined, ordinarily would have had much wider bor-

ders of inflammatory cells and relatively small compact caseous centers (2). Reinfection of the intensity used in this experiment, apparently had partially inhibited the inflammatory response of the initial tubercles and increased the extent of caseation without appreciably changing the rate at which these lesions had expanded.

In contrast to the initial tubercles in the lungs of these animals the reinfection tubercles usually were more sharply defined. The size and the extent of caseation within these lesions usually corresponded closely to initial tubercles of the same ages, but their cellular components sometimes did not. In about one-half of the animals reinfection tubercles contained appreciable numbers of epithelioid cells, whereas in the lungs of other rabbits these lesions, like initial tubercles, were composed chiefly of monocytes and leukocytes. This difference was not related to time of reinfection.

The one feature which distinguished reinfection tubercles from initial tubercles of the same age was the relatively small number of bacilli which could be demonstrated within the caseous centers. Differences between the numbers of bacilli in initial and reinfection tubercles were estimated to be of the order of one hundred to one (Figs. 4, 6, and 7). This difference did not change appreciably with the interval between exposure to infection.

One of the 3 rabbits which were exposed to reinfection 4 weeks after initial infection apparently did not develop reinfection tubercles. At autopsy of this animal, 62 days after the second exposure, the lesions found in its lungs could be attributed solely to progression of the initial tubercles. Likewise it seemed that death of all the rabbits exposed to reinfection at intervals greater than 4 weeks was the result of progressive growth of initial tubercles. Yet it was not so certain that all of them resisted completely the bacilli of reinfection. For example, one of the rabbits exposed to reinfection after 6 weeks died 21 days later. This animal, and an exposure mate which was killed 26 days after reinfection, had in their lungs numbers of small tubercles of sizes corresponding to reinfection tubercles of equivalent ages. But the kidneys of both animals also contained equally numerous small tubercles. Thus it seemed reasonable to suppose that the small tubercles in the lungs also developed from organisms which had spread by way of the blood stream from the initial tubercles.

2. Effects of Reinfection upon Progress of Initial Tubercles

In spite of the lack of convincing evidence of growth of the bacilli of reinfection or of a response to their growth when rabbits were reexposed after more than 4 weeks, the progress of the disease, in some animals of this series had been surprisingly rapid. Other instances of equally rapid progression, without evidence of developing reinfection tubercles, had occurred among rabbits which had been used in earlier experiments on reinfection. These animals had been exposed to reinfection 46 and 82 days after initial infection.

In order to allow ready comparison of the characteristics of the disease in

TABLE I

The Response of Rabbits to Massive Inhaled Reinfection at Increasing Intervals from 2 to 7 Weeks after Small Initial Inhaled Infection

Rabbit No.	Interval between infections	Time survived second infection	Character of the disease
	days	*days*	
12-0	14	20	Initial tubercles poorly outlined, largely necrotic, contain numerous bacilli; reinfection tubercles vary with the animal from well defined lesions containing epithelioid cells to poorly outlined accumulations of monocytes and leukocytes which surround necrotic foci. Bacilli scanty in all of these
12-6	"	22	
13-2	"	19	
13-8	Control	22	Tubercles poorly defined, composed of monocytes and leukocytes, and contain large necrotic foci in which bacilli are numerous
12-1	21	27 k.	Initial tubercles outlined by wide zone of monocytes, large center of necrosis; reinfection tubercles often fused but all largely cellular. Epithelioid cells, monocytes, and fibroblasts outline small necrotic centers in which there are few bacilli
12-7	"	32	All lesions largely necrotic, poorly outlined by monocytes; few bacilli in foci of reinfection
13-3	"	24	Reinfection tubercles similar to No. 12-7 but show less necrosis
13-9	Control	27	Tubercles poorly defined, composed chiefly of monocytes which outline relatively large necrotic foci in which bacilli are numerous
12-2	28	25	Initial tubercles poorly defined, largely necrotic; reinfection tubercles composed mainly of monocytes and leukocytes which form poorly outlined borders about necrotic centers. Necrosis more advanced in No. 12-2, corresponding to age of infection, few bacilli in reinfection tubercles
13-4	"	20	
12-8	"	62	No evidence of second infection. Disease limited to lungs and mucosa of the intestine
14-2	Control	24	Lesions similar to those of No. 13-9
12-3	35	86	No evidence of second infection. Subacute disease destroys much of lungs. Few small tubercles in kidneys. Mucosa of gut ulcerated

TABLE I—*Concluded*

Rabbit No.	Interval between infections	Time survived second infection	Character of the disease
	days	*days*	
13-5	35	105	Comparable to No. 12-3 but less rapidly destructive, and no evidence of spread by blood stream
12-9	35	23 k.	Initial tubercles well circumscribed, few ulcerated. Miliary tubercles scattered in lungs and kidneys. No evidence of second infection
14-6	Control	22	Lesions similar to those of No. 13-8
12-4	42	21	Initial tubercles circumscribed but excavated. Miliary tubercles numerous in lungs and kidneys. No evidence of second infection
13-6	"	26 k.	
13-0	"	50	Disease comparable to that in No. 13-6. Tubercles in kidney 3–4 mm. in diameter, gut ulcerated
14-3	Control	24	Lesions similar to those of No. 13-8
12-5	49	85	Moderate grade of blood stream spread. Disease in lungs chronic
13-1	"	121 k.	Infection limited to lungs except for few small lesions in kidneys
13-7	"	26 k.	Tubercles limited to lungs. No evidence of second infection
15-1	Control	23	Well defined small tubercles composed of monocytes and leukocytes contain small necrotic foci. Bacilli numerous

k., killed.

animals which were exposed to reinfection at intervals greater than 4 weeks, the records of 10 rabbits listed in Table I have been combined with those of 8 other animals of the 2 groups mentioned, to form Table II. In this table the animals have been arranged in the order of increasing intervals between exposures to infection. The table also shows the average number of organisms inhaled by each animal at initial infection, and the survival period following reexposure. A brief characterization of the disease in each animal is also given.

Progressive tuberculosis in the rabbits listed in Table II seemed to follow one general pattern. Certainly the rate of progress of the disease varied widely, but it involved the lungs chiefly and the length of life of the animals reflected the rapidity with which the lungs were destroyed. Hematogenous spread of

TABLE II

Survival Periods of Rabbits and the Character of Tuberculosis in Them Following Inhalation of about 20,000 Virulent Bacilli 4 or More Weeks after Initial Infection by Small Numbers of Air-Borne Tubercle Bacilli of Equivalent Virulence

Rabbit No.	Bacilli inhaled during initial exposure: approximate	Interval between exposures	Time survived second exposure	Characteristics of disease
		days	*days*	
12-8	30	28	60	Many large cavities, bronchial spread to dependent parts of lungs. No evidence of hematogenous spread
12-3	"	35	86	Large, thin walled cavities occupy greater part of lungs. Dorsal regions of lungs relatively free. Gut ulcerated and perforated
12-9	"	"	23 k.	Initial tubercles ulcerated, cavities small; many 1 mm., solid tubercles in lungs and kidneys. Extensive tuberculous pneumonia
13-5	"	"	105	Similar to No. 12-3, kidneys free
12-4	"	42	21	Similar to No. 12-9, but small tubercles in lung and kidneys 2–3 mm.
13-6	"	"	26 k.	Similar to No. 12-9
13-0	"	"	50	Initial tubercles, small, ulcerated. Many small solid, 2–3 mm. tubercles in lungs and kidneys. Tuberculous pneumonia in dependent parts of lungs; gut ulcerated
9-4	150	46	20	Similar to No. 12-4
9-2	"	"	52	Similar to No. 12-4, but less acute with few tubercles in kidneys
9-3	"	"	53	Similar to No. 12-4, without hematogenous spread
9-5	"	"	102 k.	About 75 initial tubercles 5–10 mm., about 25 ulcerated, others compact and caseous; gut widely ulcerated. Small tubercles in kidneys
13-7	30	49	26 k.	Many initial tubercles ulcerated, bronchial spread with solid tubercles clustered about bronchi, solidifying dependent parts. Kidneys free
12-5	"	"	85	Similar to No. 13-0, but less acute

TABLE II—*Concluded*

Rabbit No.	Bacilli inhaled during initial exposure: approximate	Interval between exposures	Time survived second exposure	Characteristics of disease
		days	*days*	
13-1	30	49	121k.	Few large cavities, many solid initial tubercles. Many small tubercles scattered in lungs and kidneys. Bronchial spread to dependent parts of lungs
8-5	100	82	6	Similar to No. 12-9, with more extensive tuberculous pneumonia
8-4	"	"	36	Similar to No. 12-9
8-6	"	"	40	Similar to No. 12-9
8-7	"	"	50 k.	Similar to No. 13-1

k., killed.

the infection was a frequent complication, especially of the more acute forms, but this could not be credited as a major factor in the death of any animal. Animals which exhibited chronic tuberculosis, usually had developed tuberculous enteritis also, but only one died of a perforated intestine.

The rate of progress of the disease did not appear to be related either to the length of the interval between infection and reinfection, or to the number of bacilli which were inhaled during initial infection. Instead it varied with the animal. For example the initial tubercles and the lesions associated with them were in much the same state of development in rabbits 8-4, 8-5, and 8-6, exposed to massive reinfection after 82 days, as they were in rabbits 12-4, 13-0, and 13-6 reexposed after 42 days.

In the more acute forms of the disease, as developed by rabbits 8-5, 12-4, 12-9, and 13-0, a large proportion, if not all, of the initial tubercles ulcerated. At autopsy these lesions varied from 1 to 1.5 cm. in diameter. They consisted of wide zones of inflammatory cells surrounding poorly defined cavities which were filled almost completely by soft necrotic material in which bacilli were exceedingly numerous. Masses of this material were found in adjacent bronchi. Apparently these lesions provided the intense infection of the dependent parts of the lungs which lead to the development of more or less extensive tuberculous pneumonia (Figs. 8 and 9).

The more chronic forms of tuberculosis, as illustrated by rabbits 12-8, 13-5, 8-7, and 9-5, were characterized by the development of relatively few large cavities from initial tubercles. Usually these cavities were 2 to 4 cm. in diameter, with their walls well defined by fibrous tissue. Within and about their

walls the inflammatory reaction was relatively inconspicuous and they usually contained only small amounts of exudate. Other initial tubercles in these animals apparently remained quiescent or regressed, although lesions secondary to the cavities usually were numerous in the dependent parts of the lungs (Figs. 10 and 11).

DISCUSSION

Under the conditions of these experiments the lungs of rabbits have exhibited a highly uniform response to reinfection with virulent bovine tubercle bacilli when reinfection occurred within 4 weeks after initial infection. With one exception rabbits reinfected within 4 weeks seemed to be just as susceptible to implantation of separated organisms upon alveolar walls as were normal animals. In its essential features the pattern of this response seemed to correspond closely to that of normal rabbits (1, 2). Reinfection tubercles developed at about the same rate as initial lesions, and, more often than not their histological pattern was identical with that of initial lesions. The occurrence of epithelioid cells in the reinfection tubercles of some animals was not related to time of reinfection. However, reinfection tubercles always contained far smaller numbers of bacilli than in initial tubercles of the same ages. This was the one constant difference between initial and reinfection tubercles and was no less pronounced in reinfection tubercles which developed in animals reexposed at 2 weeks than in animals reexposed at 4 weeks. Whether or not this difference in the number of bacilli resulted from a reduced rate of growth of the bacilli of reinfection, increased destruction, or from both factors has not been determined. Evidence presented by others also has been inconclusive, but has suggested, as does the present study, that the growth rate of the bacilli of reinfection was reduced (6–9).

While the apparent reduction in the growth rate of the bacilli of reinfection has not been explained, it must be taken as indicating a level of acquired resistance. It is evident that this level was developed rapidly. It was demonstrated within 2 weeks after initial infection with small numbers of virulent bacilli, inhaled as separated cells in droplet nuclei, but it did not increase further until about 4 weeks after initial infection. Then susceptibility to reinfection by methods used in this study seemed to end abruptly. Thus it may be suggested that acquired immunity of rabbits to reinfection with virulent bovine tubercle bacilli, inhaled as separated cells in droplet nuclei, corresponds in its essential features to that which develops in infections which terminate in "clinical crisis."

If, as indicated by these experiments, immunity to reinfection with virulent bovine tubercle bacilli, inhaled as separated cells in droplet nuclei, developed in slightly more than 4 weeks, then none of the bacilli of reinfection grew sufficiently to induce the development of tubercles in the lungs of animals which were exposed to reinfection at intervals of 5 weeks or more after initial infec-

tion. The present evidence is inadequate to establish this completely. In certain rabbits bacilli of reinfection may have induced the development of some of the small tubercles which were found in the lungs, although spread of organisms from initial tubercles could explain the development of these lesions. In other rabbits, however, it is evident that none of the bacilli of reinfection became established. Therefore, in spite of incomplete evidence, it is suggested that within 5 weeks after initial infection with virulent bovine tubercle bacilli, inhaled as separated cells in droplet nuclei, rabbits become immune to reinfection with these organisms.

Nevertheless some animals which were exposed to reinfection at 5 weeks or more developed more rapidly fatal disease than would have been expected of rabbits inbred for low levels of resistance to tuberculosis. Others lived as long as highly resistant rabbits which carried equal numbers of initial tubercles (10). In the more quickly fatal infections exposure to massive inhaled reinfection seemed to augment the progress of the initial tubercles which ulcerated early in their development and caused death by rapid bronchogenic spread of the infection.

It is difficult to imagine how some 20,000 separated bacilli, inhaled into the lungs and deposited upon alveolar surfaces without apparent growth, could influence the spread of the infection from scattered initial foci. However, it is certain that the progress of the initial tubercles in the lungs of rabbits reinfected before the end of the 4th week was influenced by this experience. It is perhaps possible that massive inhaled reinfection after 4 weeks so saturated the defense mechanism of some animals that, although the bacilli of reinfection were destroyed, those in the initial foci increased more rapidly than otherwise would have occurred. It is also possible that rabbits, in which rapidly progressive disease followed exposure to reinfection, had become sensitized to the aerosol by the initial infection, and the course of the disease in them was influenced by sensitization.

This phase of reinfection tuberculosis, like the details of the reaction to inhaled reinfection, seems to demand further exploration, a task for which the apparatus and techniques employed in this and earlier studies of this series are adequate.

SUMMARY AND CONCLUSIONS

At intervals from 2 to 11 weeks after normal rabbits had inhaled small numbers of virulent bovine tubercle bacilli as separated cells in droplet nuclei, groups of these animals received a single exposure to reinfection during which each animal inhaled about 20,000 separated bacilli.

Normal control rabbits which inhaled this large number of bacilli died within 4 weeks thereafter. Their deaths were attributed to destruction of the lungs by developing initial tubercles.

Eleven of 12 rabbits which were reinfected within 4 weeks after initial infection seemed to respond as normal animals. Their lungs were largely replaced

by developing reinfection tubercles when they died or were killed within 32 days after reinfection.

The inflammatory response of the reinfection tubercles was not consistently different from that of initial tubercles, although reinfection tubercles contained fewer bacilli than initial lesions of the same age.

Within 5 weeks after initial infection rabbits apparently had developed immunity to reinfection with virulent bovine tubercle bacilli inhaled as separated cells in droplet nuclei. In some of them, however, exposure to massive inhaled reinfection seemed to stimulate the progress of initial infection.

It is suggested that in rabbits the development of resistance to tubercle bacilli does not bear a linear relationship to time, but progresses in steps and within 5 weeks after small initial infection by inhalation is adequate to prevent the growth of separated bacilli when these are deposited upon alveolar walls.

It is suggested also that the basic effect of acquired resistance of rabbits to tubercle bacilli is inhibition of multiplication of the bacilli.

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EXPLANATION OF PLATES

Photography by Mr. Basil Varian, Department of Anatomy, University of Pennsylvania. Photographs are natural size. Photomicrographs are × 620. All sections from which these photomicrographs were taken were cut at one time, and stained in one lot, by one technician.

PLATE 15

FIG. 1. Dorsal view, lungs, rabbit 13-9, which died 27 days after initial infection by inhalation of some 20,000 separated bovine tubercle bacilli. These lungs are representative of the effects of massive inhaled infection. When deaths occurred earlier, tubercles were smaller and, of course, less often fused.

FIG. 2. Dorsal view, lungs, rabbit 12-2, which died 25 days after reinfection by inhalation of some 20,000 bacilli; initial infection with about 30 bacilli 21 days before reinfection. Initial tubercles seen as white spots, 5 visible. These lungs are representative of the effects of reinfection 2, 3, and 4 weeks after initial infection.

FIG. 3. A representative tubercle, rabbit 13-9. Note the numbers of bacilli in the necrotic center and the types of cells of the inflammatory zone.

FIG. 4. A representative reinfection tubercle, rabbit 12-2. This lesion seems to be identical with that shown in Fig. 3, except that bacilli are scanty in the necrotic center.

(Ratcliffe and Wells. Tuberculosis of rabbits. II)

PLATE 16

FIG. 5. A representative tubercle from the lungs of rabbit 13-8, dying 22 days after initial infection. The necrotic part of this lesion, about which the photograph centers, contains considerable numbers of bacilli either singly or in masses.

FIG. 6. A representative tubercle from the lungs of rabbit 12-6, reinfected after 14 days, in the exposure group with rabbit 13-8, and also dying 22 days after this experience.

FIG. 7. A representative tubercle from the lungs of rabbit 13-2, reinfected with rabbit 12-6. Figs. 6 and 7 illustrate variations in the cellular response found in reinfection tubercles. The cells which make up tubercles such as are illustrated by Fig. 7 correspond to initial tubercles. Lesions such as are shown in Fig. 6 resemble secondary tubercles as seen in this series of animals. Bacilli are equally scanty in the necrotic centers of these lesions.

(Ratcliffe and Wells Tuberculosis of rabbits II)

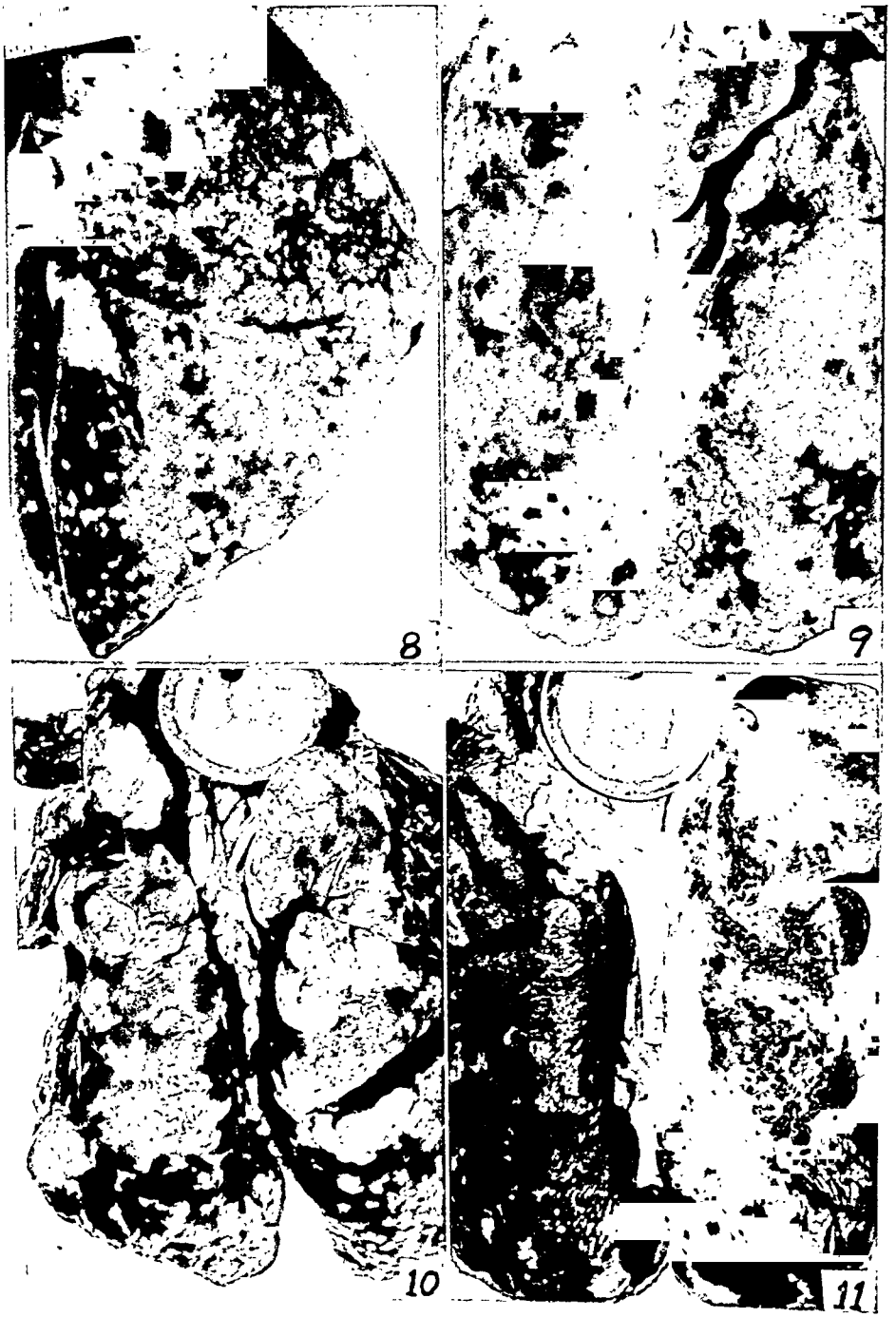
PLATE 17

FIG. 8. Lateral view, lungs, rabbit 13-7, reinfected after 49 days, killed 26 days later. Two initial tubercles are visible in dorsal parts of lungs. Dependent parts are largely occupied by secondary tubercles. Ulceration of initial tubercles into bronchi is believed to account for all small lesions. Other organs were not involved.

FIG. 9. Dorsal view, lungs, rabbit 13-6, reinfected after 42 days, killed 26 days later. Initial tubercles ulcerated, small tubercles equivalent in size to those in lungs were equally numerous in kidneys. Hence all small tubercles in lungs are believed to have developed from organisms spreading by bronchi and blood vessels.

FIG. 10. Dorsal view, lungs, rabbit 13-5, reinfected after 35 days, and dying 105 days later. Disease confined to lungs and mucosa of the intestine.

FIG. 11. Dorsal view, lungs, rabbit 13-1, reinfected after 49 days, killed 121 days later. Disease confined to lungs and mucosa of the intestine except for occasional small tubercles in kidneys. These 4 animals are believed to represent extremes of rapid and slow progression of the infection.



(Ratcliffe and Wells: Tuberculosis of rabbits. II)

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